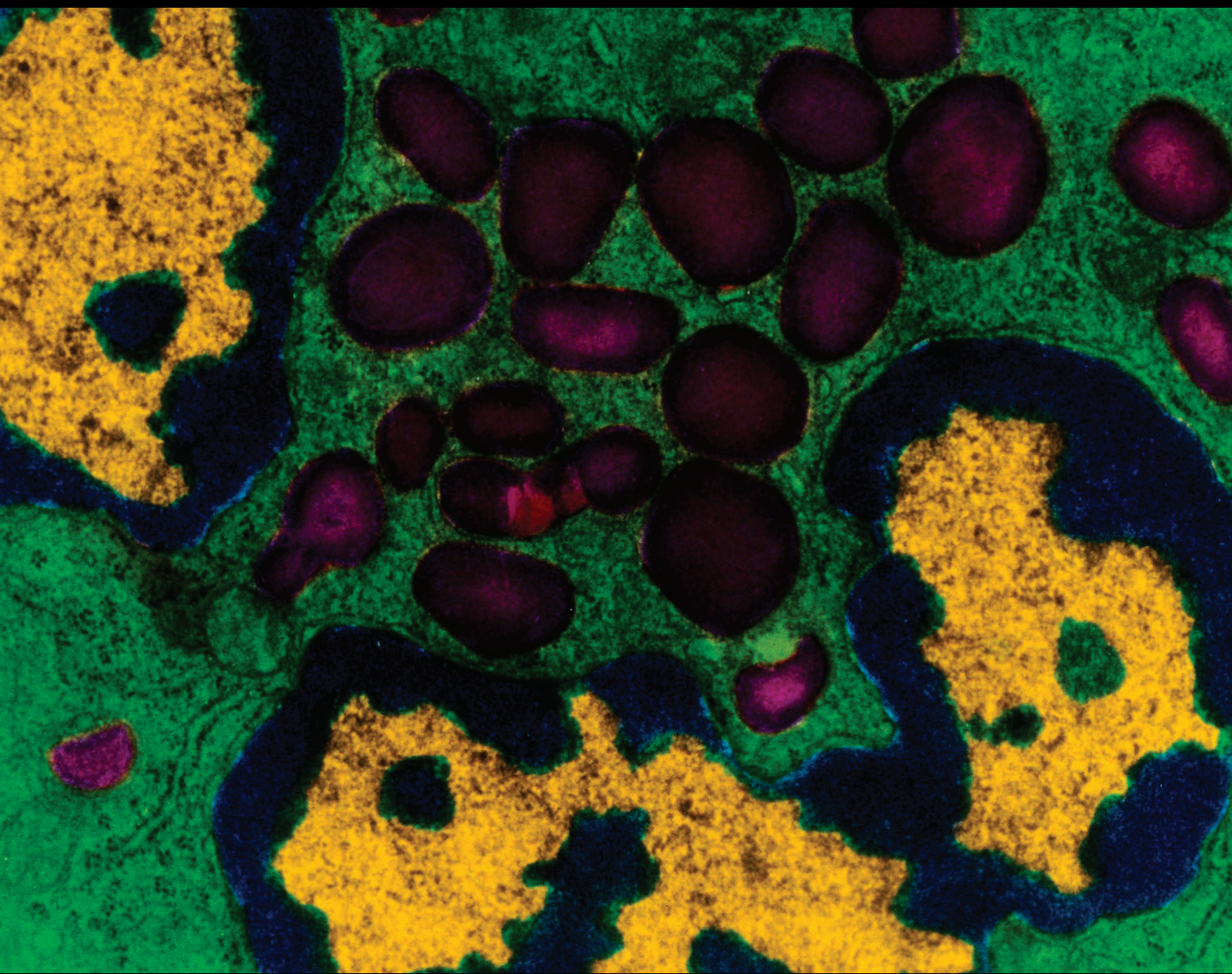
Mediators of Inflammation

Interplay between Oxidative Stress and Inflammation in Cardiometabolic Syndrome

Guest Editors: Aaron L. Sverdlov, Gemma A. Figtree, John D. Horowitz, and Doan T. M. Ngo



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Editorial Board

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Editorial

Interplay between Oxidative Stress and Inflammation in Cardiometabolic Syndrome

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Around 50–60% of adults in western countries are either overweight or obese with more than 25% falling into the obese category [1]. The metabolic imbalance underlying obesity has fueled the prevalence of cardiometabolic syndrome—a constellation of interrelated risk factors of metabolic origins that together promote the increased risk of cardiovascular disease (CVD) and type II diabetes [2]. These are the major causes of morbidity, mortality, and sky-rocketing healthcare costs in industrialized countries. Obesity is the hallmark component of cardiometabolic syndrome with other key components being insulin resistance, hypertension, dyslipidemia, and endothelial dysfunction. While each of the associated conditions has an independent effect, their clustering has a synergistic effect, making the risk of developing cardiovascular disease greater. Obesity with the associated cardiometabolic syndrome components has a direct effect on atherogenic dyslipidemia, elevated blood pressure, and elevated plasma glucose and promotes proinflammatory and prothrombotic states.

Cardiometabolic syndrome is associated with increased oxidative stress; however, the source of reactive oxygen species (ROS) and their exact targets are not well understood. Oxidation products of different organic molecules including lipids, proteins, and nucleic acids have been used to demonstrate the presence of oxidative stress in patients with cardiometabolic syndrome and CVD. Mitochondria have been shown to be a major source and target of reactive oxygen species in obesity-induced heart disease with consequences being impaired cardiac energetics, development of left ventricular hypertrophy, and diastolic dysfunction [3]. Angiotensin II-induced activation of NADPH oxidase also plays a role, with direct effects via redox posttranslational modifications of proteins within the caveolar compartment. The further downstream effects of ROS and redox regulation are mediated mostly by protein oxidative and nitrosative posttranslational modifications of proteins [4].

Cardiometabolic syndrome has also been associated with the presence of a number of inflammatory markers. Low-grade inflammation is a common manifestation and could play a role in the pathogenesis of obesity and cardiometabolic syndrome and its sequelae. Dysregulation of adipose tissue biology plays a potential role in the initiation of inflammatory events in obesity and cardiometabolic syndrome causing chronic inflammatory response characterized by abnormal adipokine production and the activation of several proinflammatory signaling pathways, resulting in the induction of several proinflammatory cytokines from adipose tissue that have been suggested to play a role in pathogenesis of CVD. Furthermore, the dysregulation adipose tissue biology, mediated by increased redox stress and inflammation, adversely affects angiogenesis both locally and systemically [5], further contributing to the global impact of cardiometabolic syndrome.

It is important to remember that cardiometabolic syndrome frequently coexists with many other diseases, linked by its high prevalence as well as commonalities in aetiology or even causative contribution. A common nexus between many of these disease states is the presence of endothelial

dysfunction, mediated either by decreased synthesis and enhanced clearance or by impaired bioaction of nitric oxide.

Rheumatoid arthritis, a common autoimmune inflammatory connective tissue condition, has been associated with increased incidence of CVD. In this issue, T. Dimitroulas et al. investigated the relationship between levels of symmetric dimethylarginine (SDMA), an inactive congener of asymmetrical dimethylarginine, a marker and mediator of nitric oxide synthase inhibition, and inflammatory burden and CVD risk factors in a cohort of rheumatoid disease patients. Interestingly, the authors have not found such an association, potentially related to the interacting effects of antirheumatoid therapy, factors affecting kinetics of SDMA and potentially methodology for SDMA measurement.

Chronic kidney disease is another condition that is intimately linked with incidence of cardiometabolic syndrome and CVD. Kidney disease can result from impaired cardiovascular function but also can in turn lead to worsening cardiac and vascular stiffening and fibrosis. A study by S. Rašić et al., in this issue, suggests that malondialdehyde (an oxidative stress marker) and matrix metalloproteinase-9 (a marker/mediator of fibrosis) are significant predictors of atherosclerosis in chronic kidney disease patients. A. Bakillah et al. looked at the levels of nitrosatively modified proteins in plasma of chronic kidney disease patients undergoing kidney transplantation and found a reduction in nitrated apolipoprotein A-I after transplantation, suggesting reduction in oxidative/nitrosative stress. In an animal model of cardiometabolic syndrome induced in rats by coca cola drinking M. Otero-Losada et al. found induction of cardiac remodeling and renal damage, associated with increase in proinflammatory cytokines, hypertriglyceridemia, and oxidative stress.

While obesity itself has been linked to increased oxidative stress and inflammation, there are many discrepancies in various observational studies. In this issue, S. M. Lee et al. found urinary malondialdehyde and CRP to be positively associated with visceral fat area in a cohort of moderately obese middle-aged men. On the other hand, weight loss has been associated with beneficial cardiometabolic remodeling, but the exact processes that lead to this improvement in metabolic and cardiovascular homeostasis are incompletely understood. S. Karki et al. observed increased expression of adipose tissue lipolytic genes following bariatric weight loss; these correlated with improvements in systemic markers of lipid and glucose metabolism, providing a potential insight into mechanisms of beneficial effects of weight loss.

Obesity and associated sedentary lifestyle are associated with an alteration in a number of secreted metabolic modulators; the precise role of many of these is still poorly understood. Irisin is a hormone secreted from the skeletal muscle under the control of PGC-1α, a metabolic master-regulator. Irisin has been suggested to be a mediator of the beneficial effect of exercise, leading to improvement of obesity and glucose homeostasis; however, some controversies regarding its role remain. M. Quiñones et al. found no difference in irisin levels with induction of obesity or manipulation of leptin levels in 2 rodent models, adding to the uncertainties regarding the role and regulation of irisin in obesity.

Poor sleep quality has been linked to development of CVD especially evident in those with disrupted sleep patterns, such as shift workers. T. Kanagasabai and C. I. Ardern investigated the relationship between sleep quality and parameters of oxidative stress and inflammation in a cross-sectional study. They found some mixed results, in general suggesting that fair quality sleepers have the most "optimal" inflammatory and oxidative profile.

Finally, high circulating levels of prothrombotic factors may play an important role in predisposing to prothrombotic episodes, which underlie cardiovascular events seen in obesity. This is further compounded by the increased incidence of atrial fibrillation seen in obese subjects, which in itself confers a much higher thromboembolic risk. N. Procter et al. studied the effect of reduction in left atrial deformation, leading to blood stasis and ultimately thromboembolic events, in atrial fibrillation. The authors did not find a relationship between reduced left atrial deformation and measures of thromboembolic risk, inflammatory activation, or platelet reactivity.

This issue on the interplay of cardiometabolic syndrome, oxidative stress, and inflammation brings together insights from a wide variety of research and disease fields. These studies add to the collective body of knowledge regarding the epidemiology, comorbidities, and mechanistic insights underlying cardiometabolic syndrome and its cardiovascular effects. The overall complexity of the pathophysiology of cardiometabolic syndrome makes these tasks difficult and overwhelming and further studies frequently add more questions than they answer. Developments in redox biomarkers for human application, currently lagging behind their inflammatory counterparts, may assist in dissecting the interaction of oxidative stress and inflammation in a more personalized fashion in the clinic and improve targeted therapeutic strategies. Increased understanding of the mechanistic processes underpinning the development and progression of the cardiometabolic syndrome will allow us to begin to improve outcomes from this syndrome which is a major contributor to mortality, morbidity, and rising healthcare costs in our society.

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Research Article

Cardiorenal Involvement in Metabolic Syndrome Induced by Cola Drinking in Rats: Proinflammatory Cytokines and Impaired Antioxidative Protection

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We report experimental evidence confirming renal histopathology, proinflammatory mediators, and oxidative metabolism induced by cola drinking. Male Wistar rats drank *ad libitum* regular cola (C, $n = 12$) or tap water (W, $n = 12$). *Measures*. Body weight, nutritional data, plasma glucose, cholesterol fractions, TG, urea, creatinine, coenzyme Q_{10}, SBP, and echocardiograms (0 mo and 6 mo). At 6 months euthanasia was performed. Kidneys were processed for histopathology and immunohistochemistry (semiquantitative). Compared with W, C rats showed (I) overweight (+8%, $p < 0.05$), hyperglycemia (+11%, $p < 0.05$), hypertriglyceridemia (2-fold, $p < 0.001$), higher AIP (2-fold, $p < 0.01$), and lower Q_{10} level (−55%, $p < 0.05$); (II) increased LV diastolic diameter (+9%, $p < 0.05$) and volume (systolic +24%, $p < 0.05$), posterior wall thinning (−8%, $p < 0.05$), and larger cardiac output (+24%, $p < 0.05$); (III) glomerulosclerosis (+21%, $p < 0.05$), histopathology (+13%, $p < 0.05$), higher tubular expression of IL-6 (7-fold, $p < 0.001$), and TNFα (4-fold, $p < 0.001$). (IV) Correlations were found for LV dimensions with IL-6 (74%, $p < 0.001$) and TNFα (52%, $p < 0.001$) and fully abolished after TG and Q_{10} control. Chronic cola drinking induced cardiac remodeling associated with increase in proinflammatory cytokines and renal damage. Hypertriglyceridemia and oxidative stress were key factors. Hypertriglyceridemic lipotoxicity in the context of defective antioxidant/anti-inflammatory protection due to low Q_{10} level might play a key role in cardiorenal disorder induced by chronic cola drinking in rats.

1. Introduction

Metabolic syndrome (MetS) is the constellation of hypertriglyceridemia, hyperglycemia, and/or insulin resistance, hypertension, and visceral obesity in man. In addition to increasing the risk for cardiovascular disease, diabetes, and diabetic nephropathy, MetS may directly affect renal morphology and/or function.

We have reported that chronic cola drinking induces MetS, pro-oxidative metabolism, and insulin resistance in rats and accelerates aortic atherosclerosis progression in adult $ApoE^{-/-}$ mice as well [1–3].

The complex heart-kidney bidirectional dialogue involves mediators which, via bloodstream in the midst of the prevailing metabolic condition, reach target tissues and deliver specific messages. We also observed that MetS induced by chronic cola drinking might also involve renal pathology in normal rats (unpublished observations). Severity of MetS, posing a major risk factor for cardiovascular disease and type II diabetes, varies depending on the number of components of the syndrome itself. Yet, the connection of MetS with risk for renal impairment is not clear. Patients with MetS are at high risk for chronic kidney disease [4]. Cardiorenal syndrome can be generally defined as a pathophysiologic disorder of the heart and kidneys whereby acute or chronic dysfunction in one organ may induce acute or chronic dysfunction in the other organ [5]. By now, this condition is

associated with significant morbidity and mortality, meeting the attention of both cardiologists and nephrologists.

Considering that cola drinking leads to metabolic changes which might individually affect heart and kidneys (e.g., severe hypertriglyceridemia and insulin resistance), the aim of this work was to evaluate whether chronic cola drinking may compromise kidney integrity in relation to oxidative metabolism and renal inflammation in rats.

2. Methods

Animal handling, maintenance, and euthanasia procedures were performed according to international recommendations [6]. The study was approved by the Committee of Ethics in Animal Research of the Instituto de Investigaciones Cardiológicas and the Institutional Animal Care and Use Committee (CICUAL) of the Faculty of Medicine of the University of Buenos Aires. Animals were housed at the institute facilities (21 + 2°C, at 12 h light-dark cycles, 7 a.m.–7 p.m.) and were fed a commercial chow (16%–18% protein and 0.2 g% sodium (Cooperación, Buenos Aires, Argentina)) *ad libitum*.

2.1. Experimental Protocol. Twenty-four male Wistar rats were randomly distributed in 2 groups, according to beverage offered as the only liquid source (*ad libitum*): W (water) or regular cola (C) (commercially available sucrose-sweetened carbonated drink, Coca-Cola™, Argentina). Food and drink consumption were assessed twice a week, body weight was determined weekly, and systolic blood pressure (SBP) was recorded biweekly. At baseline and 6 months after the beginning of the study, biochemical analyses were performed and echocardiograms (awake rats) were recorded. After 6 months of drinking treatment, all the animals were euthanized by subtotal exsanguination under anesthesia (sodium thiopental 40 mg/kg, i.p.) and kidneys were excised for histopathology and immunohistochemistry.

According to company specifications Coca-Cola is a carbonated water solution containing (approximate%) 10.6 g carbohydrates, sodium 7 mg, caffeine 11.5 mg, caramel, phosphoric acid, citric acid, vanilla extract, natural flavorings (orange, lemon, nutmeg, cinnamon, coriander, etc.), lime juice, and fluid extract of coca (*Erythroxylum novogranatense*). Cola drink had carbon dioxide content largely removed by vigorous stirring using a stirring plate and placing a magnetic bar in a container filled with the liquid prior to being offered to the animals at room temperature.

2.2. Biochemical Determinations. Plasma levels of glucose, cholesterol fractions, triglycerides (TG), urea, and creatinine were determined in blood samples collected from the tail vein after 4-hour fasting, using commercially available kits for enzymocolorimetry (Sigma-Aldrich, USA) [7]. Atherogenic index of plasma (AIP) was calculated as AIP = log (TG/HDL − total Ch).

Plasma concentration of the lipophilic antioxidant ubiquinone-10 (2,3 dimethoxy-5 methyl-6-decaprenyl benzoquinone-10, coenzyme Q_{10}) was measured using reversed phase-high performance liquid chromatography with ultraviolet detection (RP-HPLC-UV) at absorbance wavelength 275 nm [8].

2.3. Blood Pressure Determination. Systolic blood pressure (SBP) was measured by tail cuff plethysmography in awake rats gently restrained in a plastic chamber. The average of at least 3 readings per session was recorded. A pneumatic pulse transducer positioned on the ventral surface of the tail, distal to the occlusion cuff, detected the return of the pulse wave following a slow deflation of the cuff. Cuff pressure was determined by a pneumatic pulse wave transducer, using a programmed electrosphygmomanometer PE-300 connected to a Physiograph MK-IIIS for pulse recording (Narco Bio-Systems, Austin, Texas).

2.4. Echocardiography. Transthoracic echocardiograms were obtained in awake, gently restrained rats using an ATL 3000 HDI (Bethold, WA, USA) echocardiographic system equipped with a 10.5 MHz transducer. Echocardiography images (M-mode and 2-dimensional) were acquired in short axis views at the level of papillary muscle. Interventricular septal end diastolic dimension (IVSd) and left ventricular end diastolic posterior wall dimension (LVPWd) were determined at the parasternal long axis at midchordal level. Left ventricular diastolic dimension (LVDD) and left ventricular end systolic posterior wall dimension (LVPWs) were measured perpendicularly to the long ventricular axis also at midchordal level.

Typical echocardiographical parameters were calculated: shortening fraction (Sf%) = $100 \times (\text{LVPWd} - \text{LVPWs})/\text{LVDD}$; left ventricular mass (LVM) = $(\text{LVDD} + \text{RWTh} + \text{LVPWd})^3 - (\text{LVDD})^3 \times 1.04$; relative posterior wall thickness (RWTh) = (LVPWd + RWTh)/LVDD; end diastolic volume (EDV) = $0.85 \times (\text{LVDD})^3$; end systolic volume (ESV) = $0.85 \times (\text{LVSD})^3$; cardiac output = $(\text{EDV} - \text{ESV})^* \times$ heart rate; systolic volume (SV) = EDV − ESV. Echocardiographic images and heart rate (HR) were simultaneously recorded.

2.5. Histopathology and Quantitative Morphology. Kidneys were immediately dissected out after euthanasia, perfused with saline through the renal vein, weighed, and longitudinally cut. After fixation in phosphate buffered 10% formaldehyde (pH = 7.2) for 24 h, tissue pieces were embedded in paraffin, cut out into 4 μm thick sections, and routinely stained with hematoxylin-eosin (HE) and periodic acid-Schiff (PAS). Tissue sections were examined under a light microscope (Nikon Eclipse 50i, Nikon Corporation, Tokyo, Japan) for the presence of histopathological changes. Images were captured, converted to digital photomicrographs (Nikon Coolpix S4), and analyzed using the Image-Pro Plus image processing software 6.0 (Media Cybernetics, Silver Spring, Maryland, USA). Histopathological evaluation was blinded to the experimental group.

Kidney sections were classified according to the presence and severity of glomerular, tubular, vascular, and interstitial abnormalities using a semiquantitative scale from 0 (zero) indicating no alterations through 1+, 2+, 3+, and 4+ indicating mild, moderate, moderately severe, and severe

abnormalities, respectively. An overall histological score for each kidney was obtained [9].

Glomerular volume (Vg, 10^6 μm^3) was estimated based on maximal planar area (MPA) analysis which was performed using the point-counting method. An orthogonal grid with 300 test points, representing an area of 6.7 10^4 μm^2 at 40x objective lens, projected onto the fields of view. The number of points hitting the glomeruli (n) was counted in ≥50 glomeruli/kidney and used to calculate MPA (μm^2) as = $n \times d^2$, where d is between-points distance [10].

Glomerular lesions were defined by the presence of focal and segmental glomerular scarring and obliteration of glomerular capillaries with increased mesangial cellularity, mesangial matrix expansion, and adhesion formation between the tuft and Bowman's capsule. Severity of glomerulosclerosis was semiquantitatively determined by Raij's method [11].

Image analysis was performed using a Nikon Eclipse 50i microscope (Nikon Corporation, Tokyo, Japan), incorporating a digital camera (Nikon Coolpix S4) and the Image-Pro Plus image processing software 6.0 (Media Cybernetics, Silver Spring, Maryland, USA).

2.6. Immunohistochemistry. The traditional avidin-biotin-peroxidase complex technique was used and a semiquantitative score allowed determination of immunohistochemical labelling of specimens [12]. Tubular staining for thioredoxin-1 (Trx1) (TTrx1), peroxiredoxin-2 (Prx2) (T_{Prx2}), interleukin (IL)-6 ($T_{IL\text{-}6}$), and tumor necrosis factor-alpha ($T_{TNF\text{-}\alpha}$) was performed using respective primary polyclonal rabbit antibodies. Control sections were incubated with nonimmune normal rabbit serum. Intensity of immunochemistry positivity was determined by the integrated optical density (IOD) method using the Image-Pro Plus image processing software 6.0 (Media Cybernetics, Silver Spring, Maryland, USA).

2.7. Statistical Analysis. Gaussian distribution was assessed by the Kolmogorov and Smirnov method. For variables with a Gaussian distribution (parametric), values were analyzed by two-way ANOVA followed by *post hoc* tests (Bonferroni multiple t-test) in order to evaluate between-groups' differences. Pearson correlation test was used to evaluate associations between variables (SPSS™ 15.0). For variables with non-Gaussian distribution (histological scores), values were analyzed using the Kruskal-Wallis test (non-parametric analysis of variance) and Dunn's multiple comparison test for between-group comparisons. A value of $p < 0.05$ was considered significant in all cases (GraphPad Prism 5.0, GraphPad Software, Inc., San Diego, California, USA).

3. Results

After 6 months of cola drinking (C), rats showed large drinking volumes (mL/kg/24 hs) (150 ± 28 in C versus 87 ± 12 in W, $p < 0.001$) and developed overweight (+8%, $p < 0.05$), hyperglycemia (+11%, $p < 0.05$), hypertriglyceridemia (2-fold, $p < 0.001$), higher AIP (2-fold, $p < 0.01$), and lower Q_{10} levels (−55%, $p < 0.05$) compared with their water drinking counterparts (W) (Figure 1). Between-group difference in body weight increase over time became statistically significant only beyond 5 months of treatment ($p < 0.05$ at 5 months; $p < 0.01$ at 6 months, Figure 2). The decrease in Q_{10} concentration was 81% accounted for by the increase in TG and vice versa ($r = 0.90$, $p < 0.01$). Consumption of cola drinks did not modify either uremia (mg/100 mL) 33.3 ± 3 in C versus 26 ± 4 in W or creatinine (mg/100 mL: 0.53 ± 0.02 in C versus 0.58 ± 0.04, N.S.).

Echocardiographical analysis revealed that compared with W rats, C rats showed increased LV diastolic diameter (+9%, $p < 0.05$) and increased both LV diastolic volume (+26%, $p < 0.01$) and LV systolic volume (+24%, $p < 0.05$). Posterior wall thinning (−8%, $p < 0.05$) with larger cardiac output (+24%, $p < 0.05$) and no change in heart rate (HR) were also found in C rats compared with W rats (Figure 3).

Cola consumption had no effect on either HR or creatinine and did not disrupt the relationship between HR and creatinine over time (Figure 4).

Microphotographs of renal tissue revealed focal segmental glomerulosclerosis and intense tubular immunopositivity for IL-6 and TNF-α after 6 months of sustained cola drinking (Figure 5).

Cola drinking treatment induced glomerulosclerosis (+21%, $p < 0.05$), higher histopathological score (+13%, $p < 0.05$), and largely higher tubular expression of both IL-6 (7-fold, $p < 0.001$) and TNF-α (4-fold, $p < 0.001$) (Figure 6).

Correlations were found for changes in LV dimensions with IL-6 (74%, $r = 0.86$, and $p < 0.001$) and TNF-α (52%, $r = 0.72$, and $p < 0.001$). Controlling for either TG or Q_{10} values individually reduced the strength of correlations to (% of mutually explained variance) 22%, $r = 0.47$, and $p < 0.05$ for IL-6 and 14%, $r = 0.38$, and $p < 0.05$ for TNF-α. Moreover, controlling for both TG and Q_{10} levels altogether actually abolished any correlation previously observed for LV dimensions with IL-6 ($r = 0.20$, NS) and TNF-α ($r = 0.41$, NS) (Figure 7).

4. Discussion

In the present paper, the striking increase in triglycerides following regular cola consumption can be explained by high content of fructose and large drinking volumes in C group. Interestingly, the decrease in Q_{10} was 81% accounted for by the increase in TG and vice versa revealing an intimate and bidirectional metabolic connection. Hypertriglyceridemia, increasing the demand of antioxidant factors to protect against further lipoperoxidation, might be responsible for exhaustion of the mitochondrial production of Q_{10} level. Q_{10} level has been suggested to be a useful biomarker of oxidative stress [13]. In this regard, MetS is associated with higher levels of circulating oxidized LDL [14]. Cola drinking induced left ventricle hypertrophy (LVH), namely, larger diastolic and systolic volumes with posterior wall thinning, increased stroke volume, and cardiac output without affecting heart rate, likely as a result of LVH and a rise in preload (EDV) and afterload [15]. On one hand, these changes may be partly explained by fluid overload after drinking large volumes in

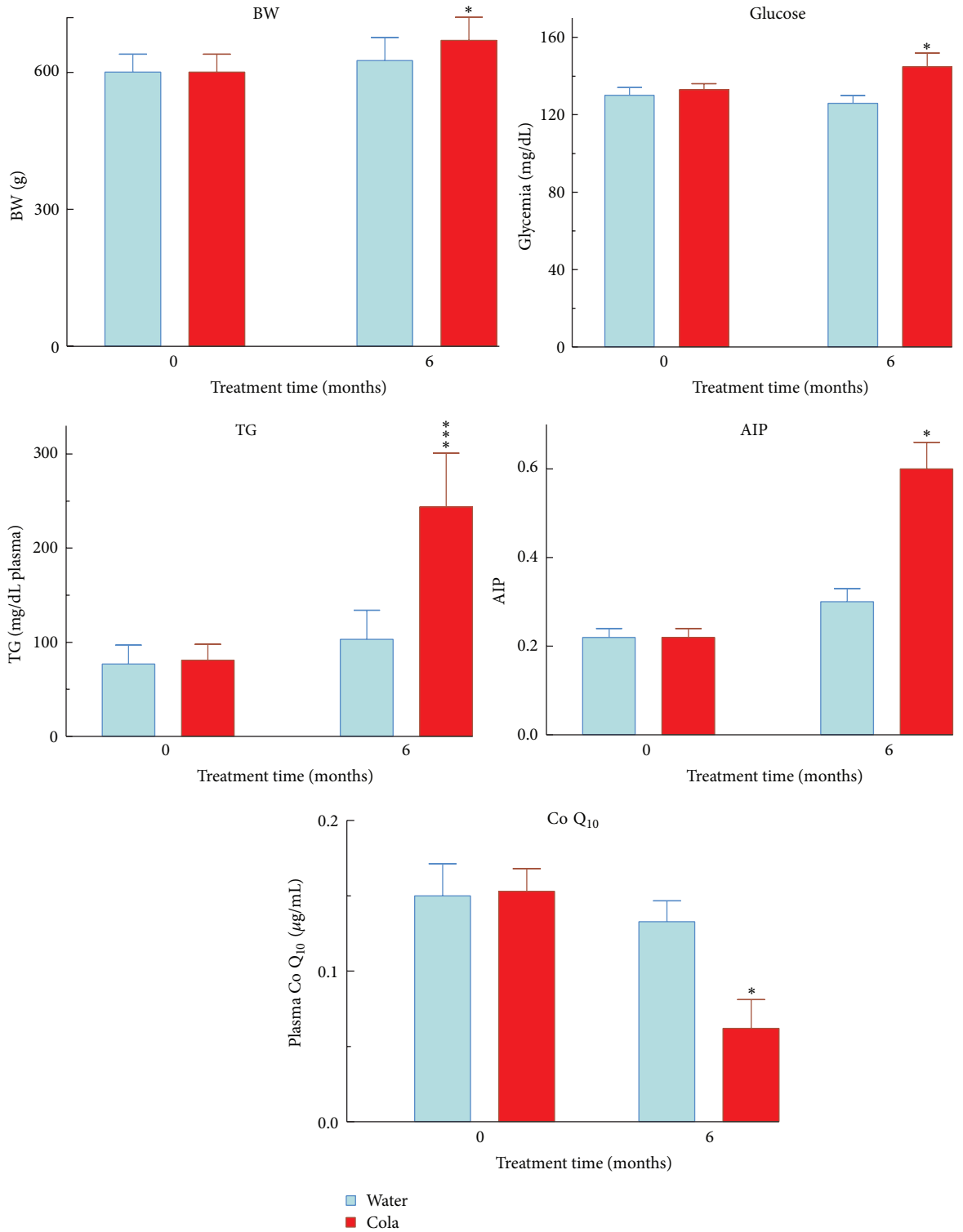

FIGURE 1: Body weight, biochemical profile (general metabolism), and Q_{10} level before and after cola treatment. BW: body weight, TG: triglycerides, AIP: atherogenic index in plasma, and Q_{10}: coenzyme Q_{10} (ubiquinone Q_{10}). $^{*}p < 0.05$ and $^{***}p < 0.001$. Compared with W, C rats showed overweight (+8%, $p < 0.05$), hyperglycemia (+11%, $p < 0.05$), hypertriglyceridemia (2-fold, $p < 0.001$), higher AIP (2-fold, $p < 0.01$), and lower Q_{10} levels (−55%, $p < 0.05$).

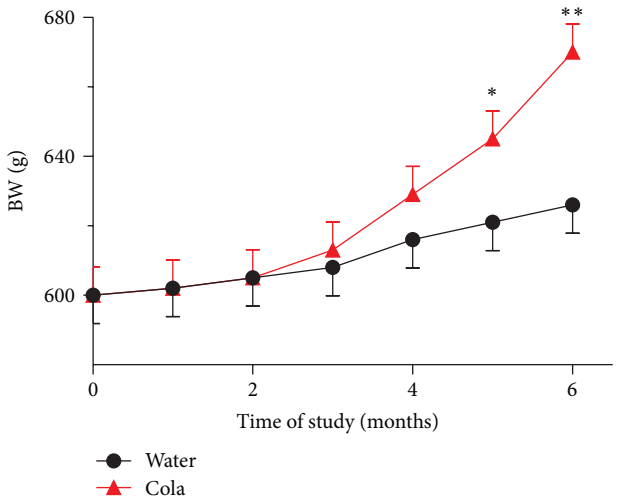

FIGURE 2: Body weight (BW) over the time of study. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus water drinking group.

C group. Ingestion of large volumes of cola, a carbohydrate-rich hypertonic solution having 493 mOsm/L compared with 285–295 mOsm/L of plasma or 3 mOsm/L in hypotonic tap water, is expected to increase blood volume and CO, through sequestration of fluid from intracellular compartments.

Cola drinking may stimulate hypothalamic antidiuretic hormone (ADH, vasopressin) secretion and increase blood volume in order to keep physiological osmolarity in plasma. The driving force responsible for the movement of fluid into the interstitial space is regulated by circulating factors, mainly glucose drawn from the splanchnic circulation [16]. Interestingly, stimulation of ADH release would stimulate thirst in C rats, helping to explain the large drinking volumes observed in this group in addition to the sweet taste preference behaviour.

On the other hand, low Q_{10} levels in plasma have been associated with cardiac hypertrophy [17]. Oppositely, Q_{10} has been reported to have a direct antihypertrophic effect on rat cardiomyocytes in vitro and combining Q_{10} with low-dose losartan provided additive therapeutic benefit, reducing hypertension and LVH [18].

Actually MetS is clearly linked to Q_{10} deficiency [19]. In experimental and human diabetic nephropathy, advanced glycation end products (AGEs) accumulate with malondialdehyde in glomerular lesions in relation to disease severity and in the presence of an upregulated receptor for AGE (RAGE) in podocytes [19]. Toxic effects of AGEs result from structural and functional alterations via cross-linking of plasma and extracellular matrix proteins. In mesangial and endothelial cells, AGE-RAGE interaction causes enhanced formation of oxygen radicals with subsequent activation of nuclear factor-kappaB and release of the proinflammatory cytokines IL-6 and TNF-α [19].

Insofar, the effects of cola intake on glomerular structure might be secondary to metabolic syndrome and/or they might be related to other factors as well, such as increased fluid overload and intravascular expansion as noted in our previous report [1]. Present results might appear as in apparent discrepancy with our earlier observations showing kidney lesions attributable to the aging process in cola drinking rats. However, thorough histopathology examination was not performed in that study.

The more we advance in the study of the effects of chronic cola drinking, the more we meet new pieces of the multifactorial puzzle upstream metabolic syndrome manifestations and long-term complications. Actually, metabolic syndrome actually poses a threat to kidney structure and function in the long run [20].

The increase in creatinine as a function of time (age of the animals) is interpreted in terms of the functional status of the kidney and its deterioration over time. Cola consumption did not affect creatinine and most important did not affect heart rate-creatinine relationship (the shape of the association curve was unaltered) suggesting that the kidney responded adequately to variation in heart rate over 6 months of cola drinking. The relationship between creatinine and heart rate has been reported [21].

On the other hand, since rats develop insulin resistance over 6 months of cola drinking as we reported [3] and present results show an increase of inflammatory mediators in renal tubules, the possibility that 6 months of cola drinking might predispose to mild renal insufficiency in due time cannot be ruled out until experimental confirmation. Mild renal insufficiency is associated with inflammation and insulin resistance [22]. Epidemiological studies have shown an association between the intake of cola beverages and chronic kidney disease [23].

In a previous study, 3-month cola drinking did not affect body weight, glomerular morphology, or oxidative status in renal cortex [23]. In contrast, in our study, mild overweight and glomerular histopathology were observed after 6-month cola drinking with no change in the immunohistochemical expression of thioredoxins, in agreement with the previous study. Cola consumption in that study (average 140 mL/day) was similar to cola intake in the present study and both studies evaluated male Wistar rats. However treatment length was largely different and, in this experimental model as in many others, changes over time are juxtaposed to changes due to time (age) itself. For instance, present difference in body weight in cola drinking rats achieved statistical significance at 5 months, not before. Hence differences between the two studies are interpreted mainly in terms of time-dependence and treatment length.

The dramatic increase in proinflammatory cytokines IL-6 and TNF-α in renal tubules induced by cola drinking rats should result in tubular derangement and dysfunction if sustained in time (not the aim of this study). Experimental and clinical studies have suggested a correlation between the progression of renal disease and dyslipidemia. Hypertriglyceridemia elicits inflammatory responses in different tissues and is known to affect morphological integrity of kidney [24]. Dyslipidemia and lipotoxicity-induced insulin resistance, inflammation, and oxidative stress are the key pathogeneses of renal damage in type 2 diabetes [25]. Interestingly, we have reported development of insulin resistance in cola drinking rats [3]. In reasonable agreement with present evidence and

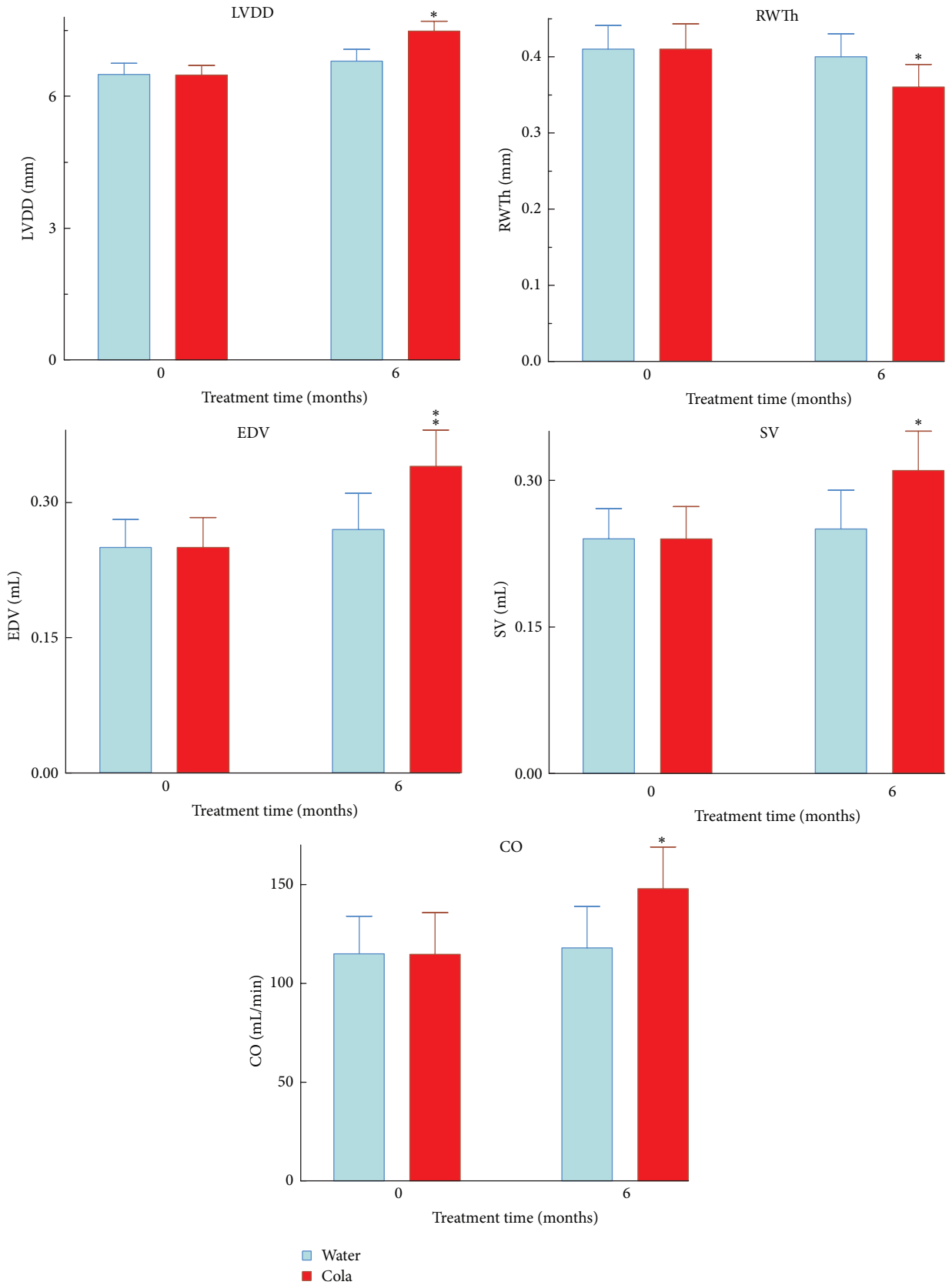

Figure 3: Echocardiographic parameters in rats before and after cola treatment. LVDD: left ventricle diastolic diameter, SV: stroke volume, RWTh: relative posterior wall thickness of LV, EDV: end diastolic volume, CO: cardiac output, and SBP: systolic blood pressure. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with water.

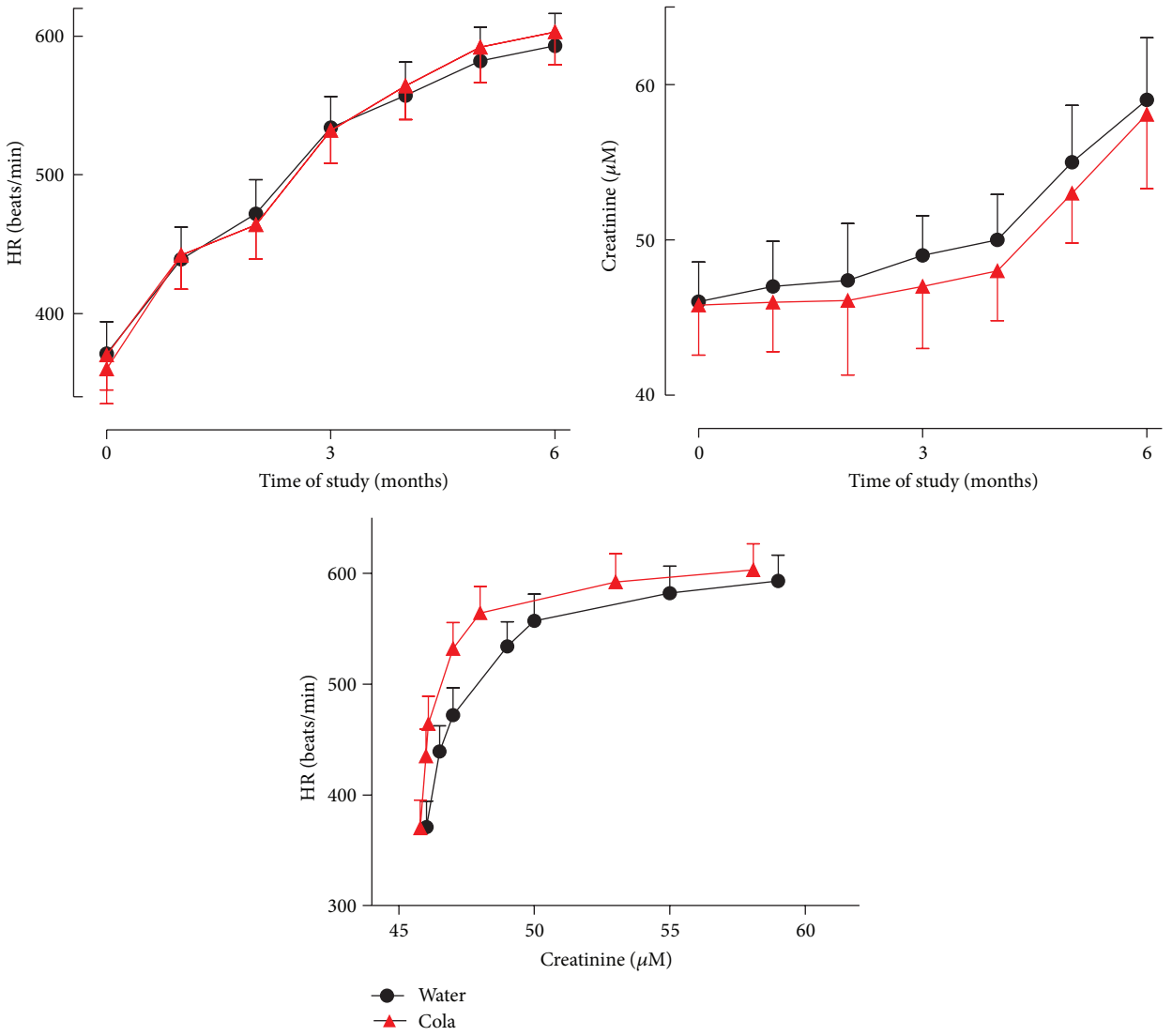

Figure 4: Heart rate (HR), creatinine, and their relationship over the time of study. Cola drinking did not affect HR, creatinine, or HR-creatinine association over time.

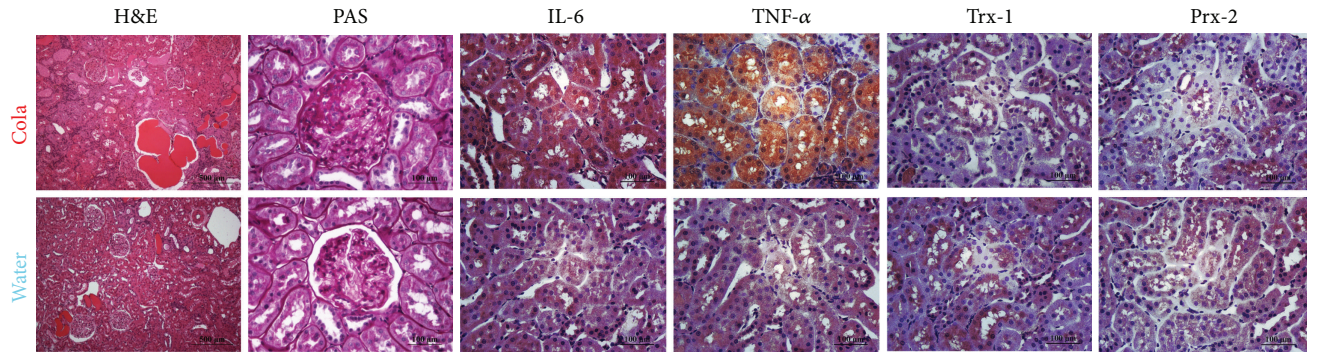

Figure 5: Immunohistochemistry of renal glomeruli and tubules before and after cola treatment. Cola treatment (C, top row) resulted in glomerulosclerosis (PAS column) and intense tubular immunopositivity for IL-6 and TNF-α, with no major change in redoxins Trx-1 and Prx-2 immunopositive labelling compared with water drinking rats (W, bottom row). H&E: hematoxylin-eosin, PAS: periodic acid-Schiff, Trx-1: thioredoxin-1, Prx-2: peroxiredoxin-2, IL-6: interleukin-6, and TNF-α: tumor necrosis factor-alpha.

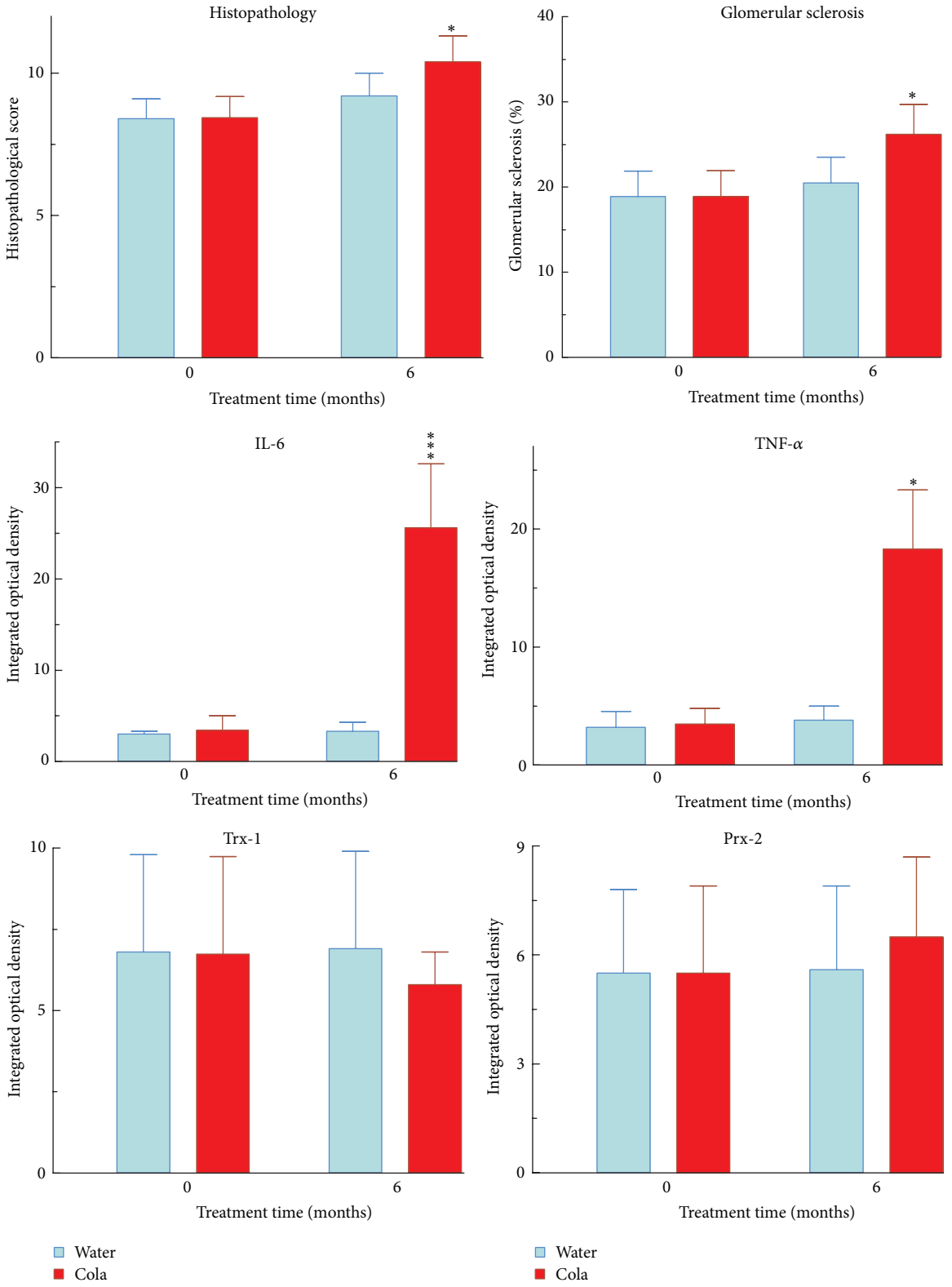

FIGURE 6: Renal glomerular pathology, inflammatory cytokines, and redoxins in renal tubules before and after cola treatment. IL-6: interleukin-6, TNF-α: tumor necrosis factor-alpha, Trx-1: thioredoxin-1, and Prx-2: peroxiredoxin-2. $^{*}p < 0.05$ and $^{***}p < 0.001$ compared with water.

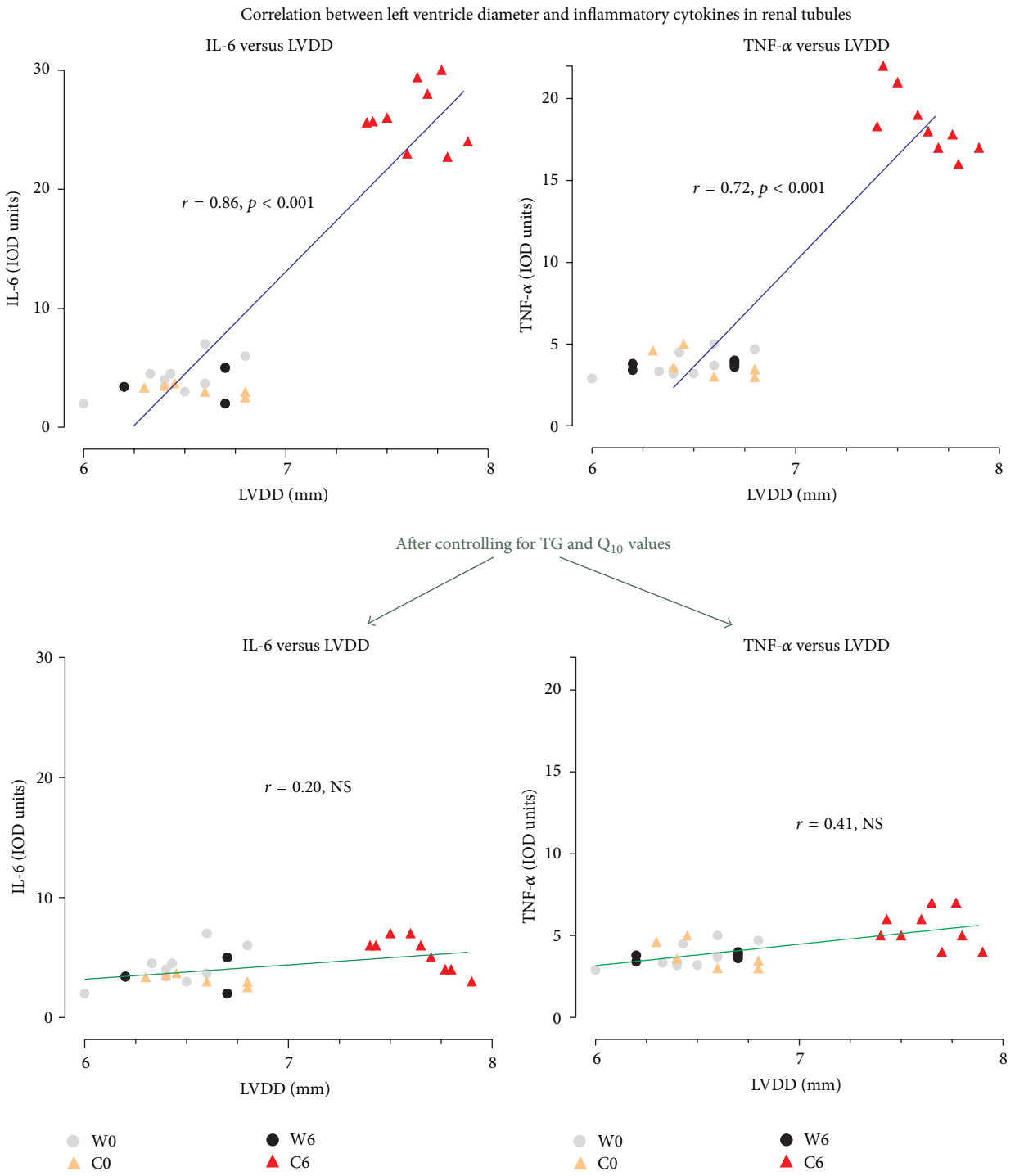

Figure 7: Cardiorenal correlations. Dependence on TG and Q_{10} levels. IL-6: interleukin-6, and TNF-α: tumor necrosis factor-alpha.

previous reports, we suggest that MetS induced by cola drinking affects kidney structure and increases proinflammatory cytokines in renal tubules in rats and that hypertriglyceridemia and low levels of Q_{10} may play crucial roles in determining the pathophysiology of the cardiorenal axis. High content of advanced glycation end products (AGEs) in caramel colorant in cola beverages further perpetuates oxidative stress, contributing to the increase in proinflammatory cytokines in renal tubules and may be involved in the progression to chronic kidney disease as one of the complications of MetS in cola beverages consumers [26].

Five subtypes of cardiorenal alterations have been identified according to pathophysiology, time-frame, and the nature of concomitant renal dysfunction. Cola drinking

might be considered to induce a metabolic condition that goes beyond the typical MetS and may progress to a type 5 cardiorenal alteration in due time (i.e., renal and cardiac dysfunction due to a systemic metabolic condition) [27].

Chronic kidney disease is an emerging health problem but only few patients would reach end renal stage. There exists an increasing strong association between MetS and chronic kidney disease though the connection between them is unclear and there are few studies showing renal histology in MetS [28]. Acute kidney injury has been recently reported in a patient with metabolic syndrome with previous normal kidney function [28]. In this paper, we present evidence showing that MetS induced by cola drinking affects renal structure in rats and increases the level of proinflammatory cytokines IL-6 and TNF-α in renal tubules, in the context of severe hypertriglyceridemia and a decrease in the antioxidant/anti-inflammatory Q_{10} levels.

5. Conclusion

Chronic cola drinking induced cardiac remodeling associated with increase in proinflammatory cytokines and renal damage. Cardiorenal association was dependent on hypertriglyceridemia and oxidative stress. Hypertriglyceridemic lipotoxicity in a context of defective antioxidant and anti-inflammatory protection due to low Q_{10} level might be involved in the cardiorenal syndrome induced by chronic cola drinking in rats.

Based on present findings and according to the classification by Ronco et al. [5], experimental MetS induced by chronic cola drinking, presenting cardiac hypertrophy and renal histopathology (glomerular sclerosis) [1, 2], may provide an interesting model to study type 5 cardiorenal syndrome as well.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Acknowledgments

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Research Article

Platelet Reactivity Is Independent of Left Atrial Wall Deformation in Patients with Atrial Fibrillation

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It has been documented recently that left atrial (LA) deformation in AF patients (while in AF) is predictive of subsequent stroke risk. Additionally, diminished LA deformation during AF correlates with the presence of LA blood stasis. Given that endothelial function is dependent on laminar blood flow, the present study sought to investigate the effect of diminished LA deformation (during AF) on platelet reactivity and inflammation in AF patients. Patients ($n = 17$) hospitalised with AF underwent echocardiography (while in AF) for determination of peak positive LA strain (LASp). Whole blood impedance aggregometry was used to measure extent of ADP-induced aggregation and subsequent inhibitory response to the nitric oxide (NO) donor, sodium nitroprusside. Platelet thioredoxin-interacting protein (Txnip) content was determined by immunohistochemistry. LASp tended ($p = 0.078$) to vary inversely with CHA_2DS_2VASc scores. However, mediators of inflammation (C-reactive protein, Txnip) did not correlate significantly with LASp nor did extent of ADP-induced platelet aggregation or platelet NO response. These results suggest that the thrombogenic risk associated with LA stasis is independent of secondary effects on platelet aggregability or inflammation.

1. Introduction

Atrial fibrillation (AF) is increasing in global prevalence and carries with it incremental thromboembolic risk. Currently, this risk is assessed through the application of clinical algorithms such as the CHA_2DS_2VASc score [1], from which appropriate antithrombotic therapy may be determined.

The precise pathophysiology underpinning the utility of the CHA_2DS_2VASc score in predicting thromboembolic events continues to be an ongoing area of investigation: one such facet of this interest is the association of inflammation with thromboembolic risk. Just as inflammation is a pivotal mediator of the pathogenesis of AF [2], it also appears to be involved in thromboembolic risk. Plasma concentrations of the inflammatory mediator Galectin-3 correlated positively [3] and those of the anti-inflammatory mediator adiponectin correlated inversely [4], with CHA_2DS_2VASc scores. The potential utility of these biomarkers in refining the prediction of thromboembolic risk continues to be explored.

An alternative approach to predicting thromboembolic risk relies ultimately on the concept that avoiding stasis of regional wall activity is protective [5]: thus the focus is to investigate the potential of extent of atrial wall motion for predictive power for incidence of thromboembolism. Recently, two-dimensional speckle tracking echocardiography was used to evaluate the extent of left atrial deformation (peak positive left atrial strain, LASp) experienced by patients in AF, correlating diminished LASp with incidence of stroke [6]. Decreased LASp has also been associated with extent of left atrial fibrosis [7], incidence of new onset AF [8], and the occurrence of left atrial blood stasis and thrombus formation [9].

However, it is also possible that the maintenance of left atrial deformation limits rheological stimuli towards thrombosis: nonlaminar blood flow has variously been associated with uncoupling of endothelial nitric oxide (NO) synthase [10], generation of reactive oxygen species [11], and increased expression of the proinflammatory mediator

thioredoxin-interacting protein (Txnip) [12, 13]. Given the aforementioned propensity for blood stasis in the presence of diminished atrial wall motility and associated risk for thromboembolism, the present study sought to investigate potential intersections of LASp and platelet reactivity, as well as the involvement of the inflammatory mediators C-reactive protein (CRP) and Txnip, in a cohort of AF patients.

2. Methods

2.1. Study Population. Patients ($n = 17$) were included as a prospectively defined subset of the Standard versus Atrial Fibrillation Specific Management Study (SAFETY) [14, 15]. Inclusion and exclusion criteria for SAFETY have been reported previously [14]. Patients receiving $P2Y_{12}$ receptor antagonist therapy were also excluded due to the impact such agents have on platelet ADP response. All patients underwent transthoracic echocardiography while in AF.

2.2. Clinical. All patients underwent routine clinical and biochemical investigation upon hospital admission. Standard echocardiography including Doppler studies was performed according to established guidelines [16]. Left ventricular ejection fraction was determined using Simpson's biplane method. Measurement of left atrial wall motion was performed as reported by Shih et al. [6], intraobserver coefficient of variation (CV) was 8.3%, and interobserver CV was 5.1%. Mean heart rate during transthoracic echocardiography was 84 ± 12 bpm.

2.3. Laboratory. Platelet aggregometry was performed using whole blood impedance aggregometry as previously described [17]. Briefly, venous blood was collected from an antecubital vein into 10 mL tubes containing 1 : 10 volume of acid citrate anticoagulant (2 parts of 0.1 M citric acid to 3 parts of 0.1 M trisodium citrate). Aggregation was induced with ADP (2.5 μM), and responses were recorded for electrical impedance (Ω) via a computer interface system (Aggrolink, Chrono-Log, Havertown, Pennsylvania, USA). The NO donor sodium nitroprusside (SNP, 10 μM) was used to measure platelet response to NO. Inhibition of aggregation by SNP was evaluated as percentage of maximal aggregation in the absence of SNP. In order to minimize inaccuracies in calculation of inhibitory effect of SNP, at least 4 Ω of ADP response was required.

2.4. Statistical Methods. All data for normally distributed parameters are expressed as mean ± standard deviation unless otherwise stated. Skewed data are expressed as median and interquartile range (IQR). Where applicable, nonnormal distributions were transformed using Log or Ln functions. Univariate correlates of LASp were sought by linear regression. Data were analyzed using the IBM SPSS Statistics 20 and GraphPad Prism 6 software packages.

3. Results

The clinical and pharmacological profiles of the study cohort can be observed in Tables 1 and 2, respectively. A total of 8 patients were excluded from analysis because of inadequate quality of LASp determination from echocardiographic records. Patients were typical for an AF population, being elderly, of moderate stroke risk on the basis of clinical scoring algorithms, and with relatively preserved renal function. The majority of patients were receiving oral anticoagulant (warfarin) therapy and renin-angiotensin-aldosterone system blockade (angiotensin receptor antagonists or angiotensin-converting enzyme antagonists), while approximately 1/3 were on lipid-lowering therapies (statins). Echocardiographic parameters of interest are summarised in Table 3.

The extent of atrial wall motility during AF bore no significant relationship with platelet reactivity (Figure 1): correlations between LASp and ADP-induced platelet aggregation, or LASp and platelet response to NO, were both nonsignificant. It should be noted, indeed, that if anything,

Table 1: Clinical profile of the study cohort.

Sociodemographic profile ($n = 17$)	
Gender, n (% male)	9 (52.9)
Age (yrs)	72 ± 12
Aged ≥ 75 years, n (%)	9 (52.9)
Comorbidities	
Congestive heart failure, n (%)	2 (11.8)
Hypertension, n (%)	11 (64.7)
Diabetes mellitus, n (%)	4 (23.5)
Prior stroke/TIA, n (%)	4 (23.5)
Clinical presentation	
Admission heart rate (bpm)	98 ± 31
Plasma creatinine (μM)	86 ± 22
Plasma CRP (mg/L)	16.0 ± 23.3
CHA_2DS_2VASc score	3.1 ± 2.0

Table 2: Pharmacological therapy present in the study cohort.

Pharmacological profile ($n = 17$)	
Antithrombotic therapy	
Aspirin, n (%)	4 (23.5)
Warfarin, n (%)	13 (76.5)
Rate and/or rhythm control therapy	
Antiarrhythmics, n (%)	5 (29.4)
β receptor antagonist, n (%)	11 (64.7)
RAAS inhibitors	
ACE inhibitor, n (%)	9 (52.9)
Angiotensin receptor antagonist, n (%)	5 (29.4)
Other medications	
Statin, n (%)	6 (35.3)

Table 3: Echocardiographic profile of the study cohort.

Echocardiographic profile ($n = 17$)	
Indexed left atrial volume ($mm \cdot m^{-2}$)	39.7 ± 13.0
Left atrial emptying fraction (%)	21.9 ± 11.0
Peak positive left atrial strain (%)	10.6 ± 2.4
Left ventricular emptying fraction (%)	53.7 ± 7.3

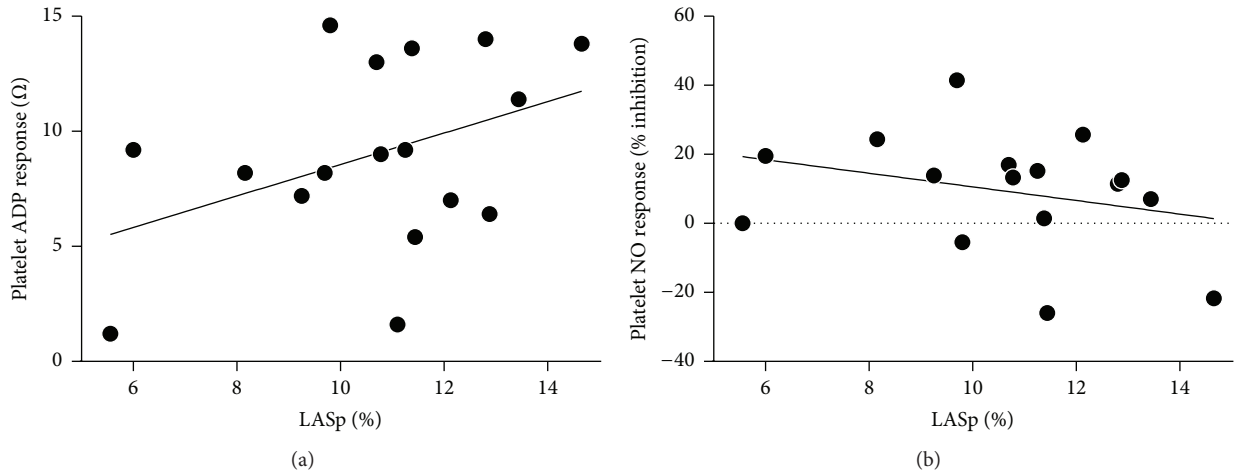

Figure 1: Relationships between peak positive left atrial strain (LASp) and (a) ADP-induced platelet aggregation ($r = 0.408$, $p = 0.104$) or (b) platelet response to NO ($r = -0.290$, $p = 0.275$).

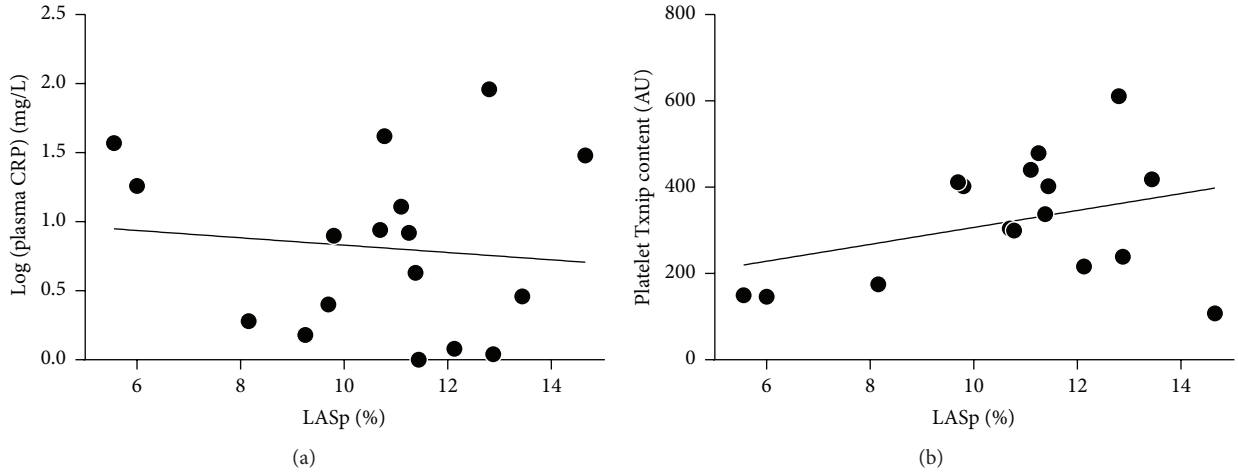

Figure 2: No significant correlation was observed between LASp and (a) plasma CRP concentrations ($r = -0.105$, $p = 0.689$) or (b) platelet Txnip content ($r = 0.345$, $p = 0.191$).

there was an association ($p = 0.104$) between increasing LASp and increasing ADP-induced platelet aggregation. Similarly, LASp was not significantly associated with plasma CRP concentrations nor with platelet Txnip content (Figure 2). A trend towards an inverse association between CHA2DS2VASc scores and LASp was observed, while CHA2DS2VASc scores and indexed LA volume were directly correlated (Figure 3).

4. Discussion

The current study, unique in the literature to date, has suggested that LASp may vary inversely with clinical indices of stroke risk, such as CHA_2DS_2VASc score. However, it has failed to demonstrate any evidence that maintained LASp might protect against thromboembolism by restoring homeostasis as regards the inflammatory and autacoid bases for thrombosis.

Patients with AF are particularly susceptible to the development of thrombi in their left atrium, with the presence of left atrial blood stasis, a significant factor in their formation [18, 19]. Similarly, left atrial stasis has been correlated with clinical risk factors and stroke incidence [5, 20]. Such thrombi are commonly platelet-rich in their morphology [21]; while not correlated with clinical measures of thromboembolic risk [22, 23], the platelet hyperaggregability that is present in patients with AF [24, 25] nonetheless would seemingly have an integral role in the formation of such thrombi.

One of the factors contributing to platelet hyperaggregability in this context is potential impairment of the NO signaling pathway (see [26] for review). Effective generation of NO by endothelial NO synthase is impaired by the presence of nonlaminar blood flow, such as what occurs in the presence of AF [11, 27, 28]. In theory, the extent of atrial motility present during AF may help preserve the functioning of this pathway through amelioration of blood stasis and, as such, would be

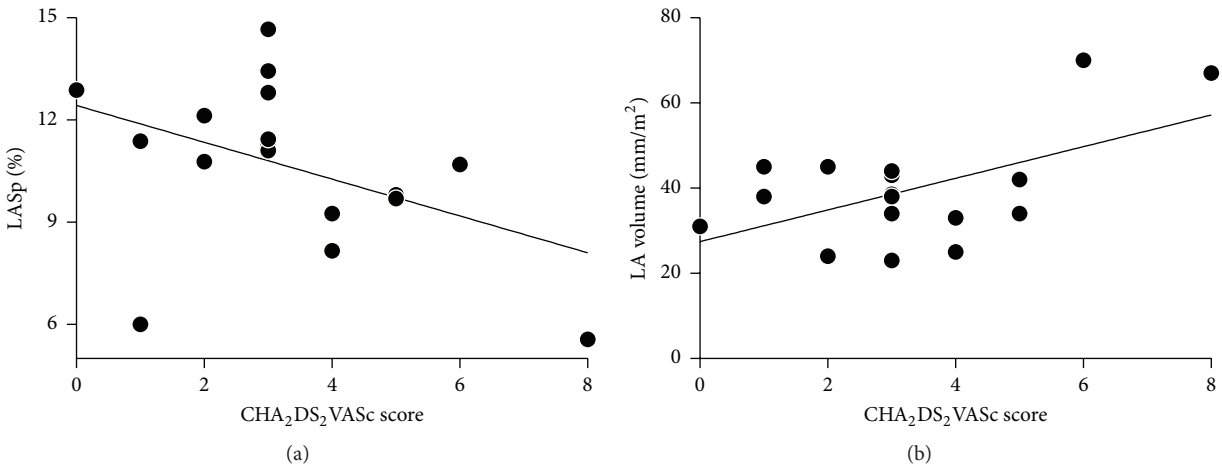

Figure 3: (a), An inverse trend between LASp and CHA_2DS_2VASc score was nonsignificant ($r = -0.44$, $p = 0.08$). (b) CHA_2DS_2VASc score and indexed LA volume were directly correlated ($r = 0.56$, $p < 0.05$).

reflected through measures of platelet reactivity. However, in our investigation we observed no such correlation between atrial wall movement and platelet response to ADP or NO. Similarly, inflammation is regulated in part by NO [29, 30]; specifically Txnip expression is suppressed by the presence of NO [31, 32]. Yet no correlation was observed between atrial wall motion and markers of inflammation in the current investigation.

One potential cause for error in acquisition of LASp data is variable degrees of tachycardia in the LA [6]. This problem was minimized because the actual heart rate during LASp measurement was 84±12 beats per minute (bpm) and because multiple views of the LA were incorporated. Reproducibility of LA strain estimates in AF patients is best achieved by considering only periods of relative heart rate stability [33]. No particular precautions were taken in this regard, and this therefore constitutes a potential limitation on accuracy of data.

The major limitation of the current study is the potential for type 2 error. However, it seems unlikely from the current data that any strong correlation has been missed, as regards the measured physiological parameters. However, it is possible that platelet responsiveness to NO may not reflect that of the endocardial endothelium.

According to the principles outlined by Virchow's triad [34], the presence of blood stasis in the atria during AF is a predisposing factor for thrombogenesis. The current data dissociate this stasis from increased platelet reactivity and/or inflammation. Given that biomechanical (such as LASp), as opposed to biochemical, factors are the primary determinants of left atrial thrombogenesis, it would be expected that effective maintenance of sinus rhythm in AF patients would significantly reduce their thromboembolic risk through improved atrial flow.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

Effect of Bariatric Weight Loss on the Adipose Lipolytic Transcriptome in Obese Humans

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Background. Dysregulated lipolysis has been implicated in mechanisms of cardiometabolic disease and inflammation in obesity. *Purpose*. We sought to examine the effect of bariatric weight loss on adipose tissue lipolytic gene expression and their relationship to systemic metabolic parameters in obese subjects. *Methods/Results*. We biopsied subcutaneous adipose tissue in 19 obese individuals (BMI 42 ± 5 kg/m^2, 79% female) at baseline and after a mean period of 8 ± 5 months (range 3–15 months) following bariatric surgery. We performed adipose tissue mRNA expression of proteins involved in triglyceride hydrolysis and correlated their weight loss induced alterations with systemic parameters associated with cardiovascular disease risk. mRNA transcripts of adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and lipid droplet proteins comparative gene identification 58 (CGI-58) and perilipin increased significantly after weight loss ($p < 0.05$ for all). ATGL expression correlated inversely with plasma triglyceride (TG), hemoglobin A1C (HbA1C), and glucose, and HSL expression correlated negatively with glucose, while CGI-58 was inversely associated with HbA1C. *Conclusion*. We observed increased expression of adipose tissue lipolytic genes following bariatric weight loss which correlated inversely with systemic markers of lipid and glucose metabolism. Functional alterations in lipolysis in human adipose tissue may play a role in shaping cardiometabolic phenotypes in human obesity.

1. Introduction

Obesity and its associated widespread metabolic abnormalities such as insulin resistance and dyslipidemia have emerged as major public health problems worldwide [1, 2]. Among the multiple mechanisms responsible for mediating obesity-related cardiovascular disease is the upregulated concentrations of circulating free-fatty acids (FFA) that have been associated with insulin resistance and inflammation [3–5]. Fatty acids play important physiological roles in energy metabolism while also serving as signaling molecules, and their mobilization from triglycerides (TG) is regulated by specific hydrolytic lipases including adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL), as well as lipid droplet proteins comparative gene identification protein 58 (CGI-58) and perilipin.

Experimental studies suggest that functional alterations in the enzymatic activity of lipases under obese conditions may lead to dysregulated FFA metabolism. ATGL protein and mRNA are downregulated in mouse models of obesity [6]. Overexpression of ATGL specific to adipose tissue promotes fatty acid use and attenuates diet-induced obesity [7], while ATGL deficiency leads to changes in insulin signaling and ectopic fat accumulation in nonadipose tissue [8–10]. However, expression of lipases in human obesity is incompletely studied [11–13]. One report demonstrated that both lean and obese subjects express comparable amounts of ATGL protein while HSL is attenuated in obesity [11]. Conversely, another study suggested that although obese subjects express significantly high ATGL mRNA, protein expression is reduced. Moreover, they reported that HSL mRNA was upregulated in visceral but not subcutaneous fat in obesity [12]. Another

investigation observed significant reductions in both ATGL and HSL mRNA and protein in obese insulin resistant subjects, and weight loss by hypocaloric diets induced decreases in ATGL and HSL expression [13]. The literature thus demonstrates variable transcriptomic signatures in largely cross-sectional comparisons between different groups of individuals, and limited data are available on longitudinal effects of weight reduction.

Bariatric surgery is currently the most effective and durable method for sustained weight loss and cardiometabolic benefit for the treatment of obesity [14–16]. In the present study, we sought to examine the effect of bariatric surgery on adipose tissue expression of lipolytic enzymes before and after extensive weight loss in obese humans and determine whether tissue changes associate with systemic markers of whole body metabolism.

2. Materials and Methods

2.1. Study Subjects. Consecutive obese men and women (BMI $\geq 35\,kg/m^2$, age ≥ 18 years) with long-standing obesity enrolled in the Boston Medical Center Bariatric Surgery Program were recruited into the study. Subcutaneous adipose tissue samples at baseline were collected intraoperatively from the lower abdominal wall during planned bariatric surgery, as previously described [17–19]. Follow-up fat tissue biopsy was performed percutaneously via periumbilical punch and needle biopsy of subcutaneous fat during a postoperative follow-up visit. The subcutaneous depot that is sampled intraoperatively is the same anatomic layer that is accessed in our follow-up transcutaneous biopsy. Each subject provided two biopsy specimens from the subcutaneous depot, one at baseline and one during the postoperative visit. All biopsies were performed under fasting conditions. Subjects with unstable medical conditions such as active coronary syndromes, congestive heart failure, systemic infection, acute illness, malignancy, or pregnancy were excluded. The study was approved by the Boston University Medical Center Institutional Review Board and written consent was obtained from all participants.

2.2. Percutaneous Adipose Tissue Biopsy. For follow-up adipose tissue biopsies, subjects were placed in supine position with sterile draping of the abdominal region. Local skin anesthesia was performed with subcutaneous lidocaine injection and a small superficial 0.5 cm skin incision made lateral to the umbilicus with a tiny scalpel which allows for both aspiration of fat using a large-bore cannula and several punch biopsies and/or manual debridement of tissue below the skin layer, providing specimens of intact adipose tissue. The anatomic layer and qualitative yield of this procedure are the same as the intraoperative baseline collection. The superficial skin incision was then closed with self-absorbing sutures and biopsy sites were inspected in follow-up clinic within 1 week.

2.3. Anthropometric and Biochemical Measures. During a presurgical outpatient and subsequent postoperative follow-up visit, clinical characteristics including blood pressure, height, weight, body mass index (BMI), and waist circumference were measured, and cardiovascular risk factors were recorded. Fasting blood was taken via an antecubital vein for biochemical measures including lipids, glucose, insulin, glycosylated hemoglobin (HbA1c), high-sensitivity CRP (hs-CRP), and homeostasis model assessment (HOMA) as the index of insulin sensitivity. All biochemical analyses were performed by the Boston Medical Center clinical chemistry laboratory.

2.4. Adipose Tissue Gene Expression. Immediately following adipose tissue collection, tissue samples were stored in RNAlater (Sigma Aldrich) solution at −80°C. Total RNAs were isolated from homogenized whole adipose tissues using the QIAzol reagent and RNeasy Mini kits (Qiagen, Germantown, MD). RNA (0.5–1.5 μg) was retrotranscribed with High Capacity cDNA Synthesis Kits (Life Technologies). Quantitative real time PCR reactions were performed using TaqMan gene expression assays in a ViiA7 PCR system (Life Technologies). Results were analyzed with the $\Delta\Delta$Ct method using GAPDH as a reference.

2.5. Statistics. Clinical characteristics of subjects were analyzed using SPSS 20.0 and presented as mean ± SD or percentage. All other analyses were performed using GraphPad Prism 6.0 software. Differences in clinical characteristics and gene expression between baseline and follow-up visits were examined using Student's paired t-tests. Spearman correlation analysis was performed to examine associations between lipolytic gene expression and clinical parameters which were normally distributed. A value of $p < 0.05$ was accepted as statistically significant. Graphic data are presented as mean ± SEM unless otherwise indicated.

3. Results

3.1. Clinical Characteristics. A total of 19 obese (BMI 42 ± $5\,kg/m^2$, 79% female) subjects were enrolled and followed longitudinally for a mean period of 8 ± 5 months (range 3–15 months) after bariatric surgery. Table 1 displays the clinical characteristics of subjects at baseline and after weight loss. As expected, bariatric intervention produced a significant 25% weight decline for the entire group. This was associated with significant decreases in BMI, waist circumference, HbA1c, triglycerides, HOMA-IR, insulin, glucose levels, and prevalent hypertension. Additionally, there was a marked decrease in hs-CRP as a marker of systemic inflammation with >5-fold decline following bariatric surgery.

3.2. Adipose Tissue Gene Expression. As shown in Figure 1, relative mRNA expression of ATGL (a), HSL (b), CGI-58 (c), and perilipin (d) significantly increased after weight loss surgery compared to baseline, with greatest relative increase observed for perilipin.

3.3. Correlations between Lipolytic Gene Expression and Clinical Parameters. Plasma concentration of TG (Figure 2(a)), HbA1C (Figure 2(b)), and glucose (Figure 2(c)) correlated negatively with ATGL mRNA expression after weight loss. In addition, plasma glucose was inversely associated with HSL mRNA (Figure 3(a)), with a similar finding trending for HbA1C ($p = 0.08$, data not displayed). Gene expression of

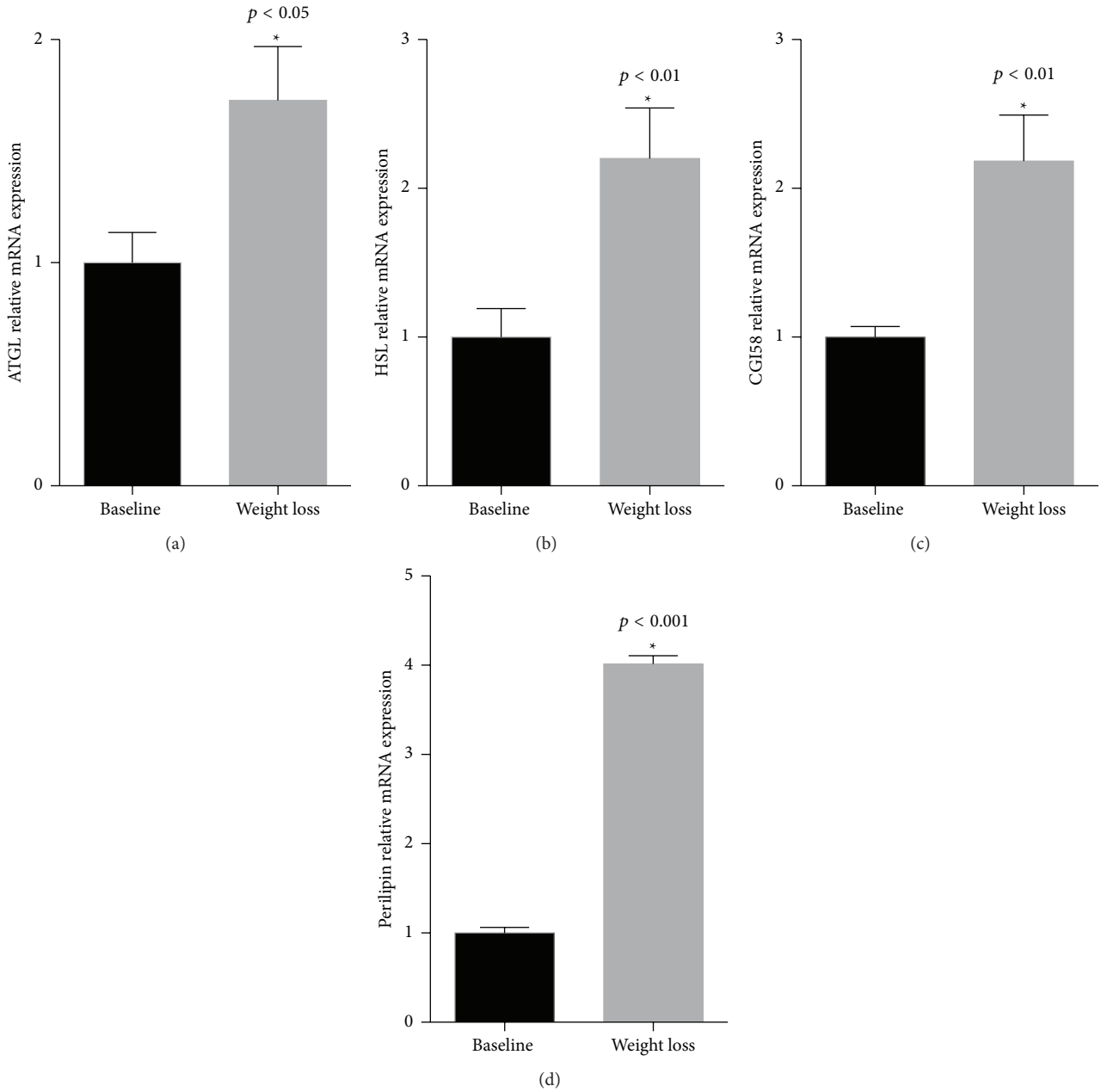

Figure 1: Lipolytic gene expression before and after bariatric surgery. Lipolytic gene mRNA for ATGL ((a), $p < 0.05$), HSL ((b), $p < 0.01$), CGI-58 ((c), $p < 0.01$), and perilipin ((d), $p < 0.001$) significantly increased in subcutaneous adipose tissue following bariatric surgery. Data are presented as relative expression, mean ± SEM, $n = 19$.

CGI-58 gene was inversely correlated with plasma HbA1C after weight loss (Figure 3(b)). We found no correlations between adipose gene expression and clinical parameters at baseline (data not displayed).

4. Discussion

In the present study, we longitudinally examined the effect of surgical weight loss on subcutaneous adipose tissue transcripts of lipases and lipid droplet proteins involved in triglyceride hydrolysis and metabolism in obese humans. mRNA transcripts of ATGL, HSL, CGI-58, and perilipin significantly increased following weight loss, and their expression correlated inversely with systemic metabolic parameters including plasma triglycerides, glucose, and HbA1C. These findings suggest that weight decline is associated with lipolytic alterations that are detectable in human adipose tissue and are linked to processes that may regulate systemic metabolism.

Adipose tissue serves as an energy reservoir that modulates triglyceride clearance and FFA release in response to whole body metabolic requirements. Under conditions of obesity and positive energy balance, fat accumulates in

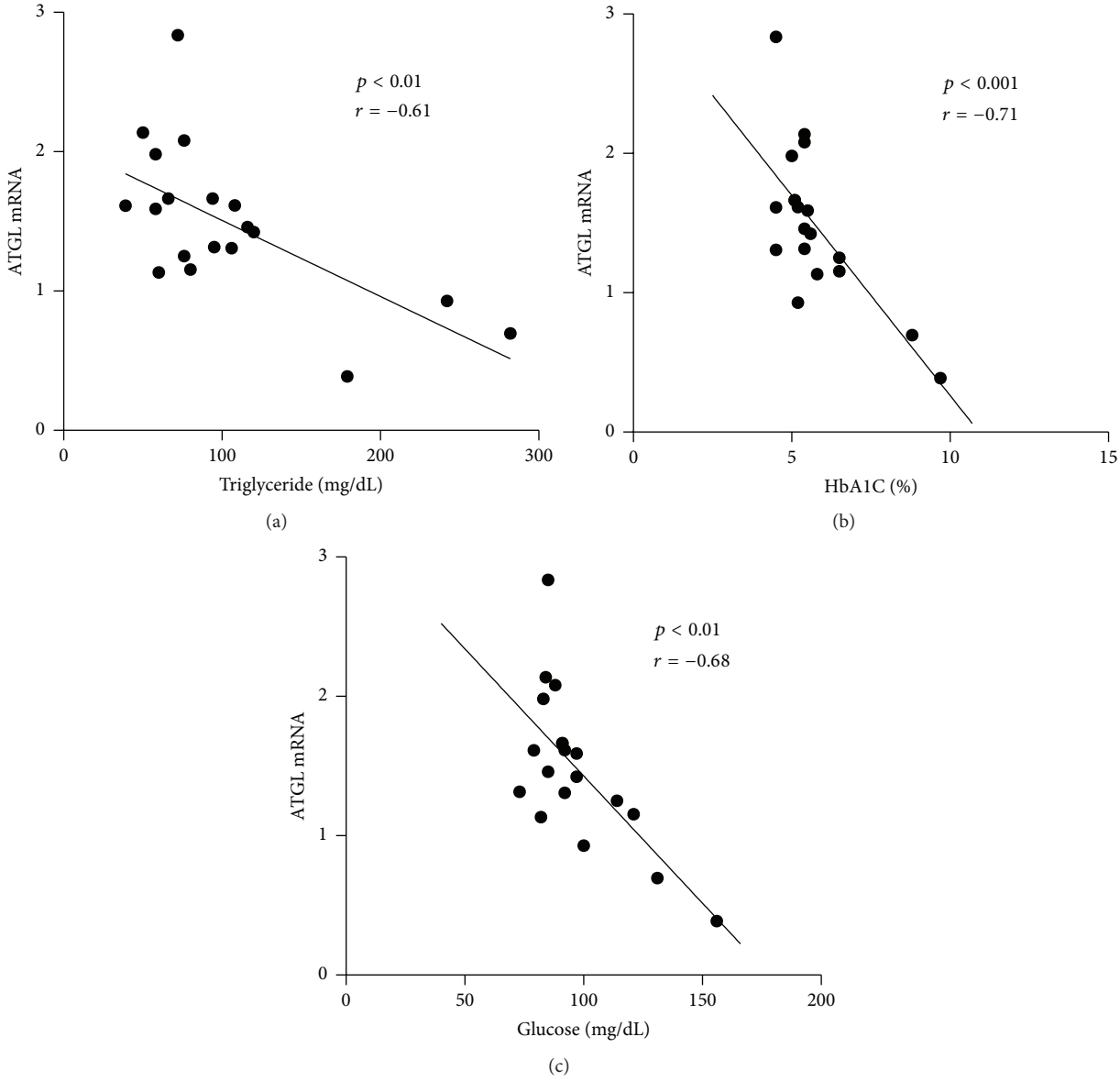

Figure 2: Correlations between ATGL mRNA and clinical parameters. (a) ATGL mRNA expression inversely correlated with plasma triglycerides after weight loss ($p < 0.01$, $r = -0.61$). (b) ATGL mRNA inversely correlated with plasma HbA1C ($p < 0.001$, $r = -0.71$). (c) ATGL mRNA was negatively associated with fasting blood glucose ($p < 0.01$, $r = -0.68$).

both adipose tissue and ectopic organs and is associated with the development of dyslipidemia, insulin resistance, and inflammation in both animal models and humans [17, 20–23] which increase cardiovascular disease risk. These processes may involve dysregulation of several enzymes including ATGL and HSL and cofactors. Although the published literature is mixed on the relative expression of ATGL in human adipose tissue in obesity [11–13, 24], we demonstrated consistent increases in expression following weight loss which suggests downregulation under obese conditions. Weight decline would presumably stimulate lipolysis and thus increase enzymatic activity in human fat stores. Moreover, we observed that this process is associated with improved insulin resistance possibly owing to decreased FFA flux, as ATGL has been linked with increased insulin sensitivity [25] and increased plasma FFA mobilization [26].

Data on HSL activity and expression in obesity have also been mixed. There are reports of decreased HSL lipolytic activity with obesity [11], and possible gender differences with decreased or unaltered HSL protein with weight loss [27, 28]. We now demonstrate consistent increases in HSL mRNA, as with ATGL, following weight loss which correlated inversely with plasma glucose. We also observed upregulation of perilipin and CGI-58 which represent key proteins associated with intracellular lipid droplets. Mutations in CGI-58 in animal models lead to deficient catabolism of cellular triacylglycerol and promote lipid accumulation in nonadipose tissue [29]. Moreover, patients with mutations in CGI-58

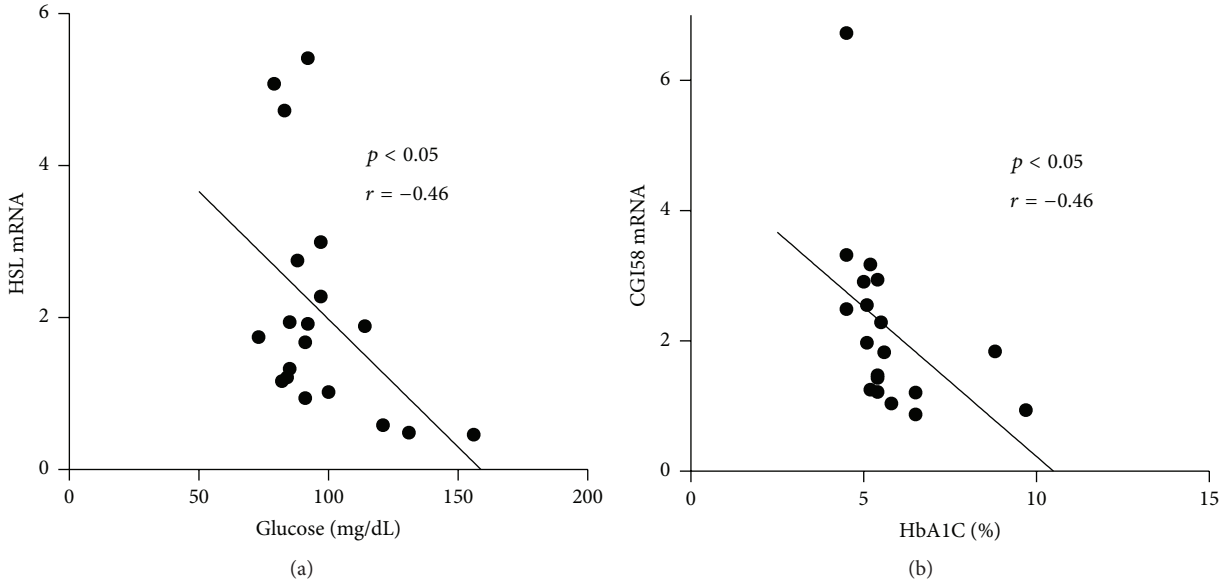

Figure 3: Associations between HSL, CGI-58 mRNA, and clinical parameters. (a) HSL mRNA expression negatively correlated with fasting blood glucose level after weight loss ($p < 0.05$, $r = -0.46$). (b) CGI-58 mRNA was negatively associated with HbA1C ($p < 0.05$, $r = -0.46$).

Table 1: Clinical characteristics.

Parameter	Baseline	Weight loss	p value
BMI (kg/m^2)	42 ± 5	32.2 ± 6	<0.001
Waist circumference (cm)	123 ± 12	91 ± 37	<0.01
Weight (kg)	114 ± 14	85 ± 14	<0.001
Insulin (mIU/mL)	26.6 ± 34	11.4 ± 14	<0.001
Glucose (mg/dL)	139 ± 99	98 ± 21	<0.05
HbA1C (%)	6.6 ± 2.3	5.8 ± 1.4	<0.05
HOMA-IR	4.6 ± 8.4	3.1 ± 5	<0.05
hsCRP (mg/dL)	10.6 ± 9.7	2.0 ± 2	<0.05
Triglycerides (mg/dL)	119 ± 81	91 ± 37	<0.05
HDL-C (mg/dL)	51 ± 14	56 ± 16	0.31
LDL-C (mg/dL)	117 ± 36	102 ± 34	0.12
Systolic BP (mmHg)	126 ± 14	127 ± 15	0.66
Diastolic BP (mmHg)	73 ± 14	77 ± 11	0.71
Diabetes (%)	21	11	0.07
Hypertension (%)	40	13	<0.05
Hypercholesterolemia (%)	16	5	0.08

Data are mean ± SD. $n = 19$.

exhibit defective lipolysis and ectopic lipid accumulation in multiple tissues [30]. It is believed that efficient ATGL activity requires CGI-58 which associates with lipid droplets and interacts with perilipin that modulates droplet turnover [31]. Our observation of a coordinate upregulation of all four measured transcripts associated with lipolysis and lipid droplets following weight loss suggests that their activities may be interlinked for effective triglyceride hydrolysis and consequently whole body metabolism, as supported by significant correlations with systemic measures of triglyceride and glucose handling.

In the present study, we also observed a remarkable decline in plasma hs-CRP which has been described previously in association with weight loss [32, 33]. It is well established that obesity is associated with a chronic, subclinical degree of inflammation that is derived in part from macrophage-driven adipose tissue inflammation in response to several pathological tissue changes including adipocyte hypertrophy [21, 34]. Local overproduction of FFA may also represent a mediator for immune activation [3, 4, 35, 36] and our observation of reduced inflammation with weight loss may be related, in part, to decreased FFA following weight loss as previously established [37, 38], although this was not specifically measured in this study.

There are several limitations to our study. The sample size is relatively small and experimental design was limited to a surgical population undergoing bariatric surgery and thus findings may not be applicable to the general population or lesser degrees of obesity. However, our demonstration of significant clinical correlations even with this small sample makes our results more compelling. Moreover, our findings are limited to the subcutaneous depot. We acknowledge that the visceral depot may be more metabolically active compared to subcutaneous one; however sequential visceral biopsies are not possible since a repeat invasive abdominal operation would be required which is not justifiable for only research purposes. However, we believe that much can be learned from examining longitudinal changes in the subcutaneous fat of obese individuals. Additionally, we acknowledge that methods for collecting fat samples at baseline and follow-up biopsy were different and may have affected gene expression; however we believe that the techniques which are all performed under fasting conditions are comparable. We did not measure protein levels or examine functional activity of these enzymes; thus observations are limited to mRNA expression patterns; however protein tracked mRNA

transcripts in a prior study [13]. Lastly, our study design does not enable us to distinguish between the effect of negative energy balance and the effect of weight loss on changes in gene expression and clinical parameters. These limitations are counterbalanced by our ability to study the same subjects longitudinally by examining the effect of major weight loss and studying changes in adipose tissue that may provide clues to mechanisms of systemic disease.

In conclusion, we observed increased expression of adipose tissue lipolytic genes which correlated inversely with systemic markers of lipid and glucose metabolism following bariatric weight loss. Functional alterations in lipolytic activity in human adipose tissue may play important roles in shaping systemic phenotypes associated with cardiovascular risk factors in human obesity.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Plasma Nitration of High-Density and Low-Density Lipoproteins in Chronic Kidney Disease Patients Receiving Kidney Transplants

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Background. Functional abnormalities of high-density lipoprotein (HDL) could contribute to cardiovascular disease in chronic kidney disease patients. We measured a validated marker of HDL dysfunction, nitrated apolipoprotein A-I, in kidney transplant recipients to test the hypothesis that a functioning kidney transplant reduces serum nitrated apoA-I concentrations. *Methods*. Concentrations of nitrated apoA-I and apoB were measured using indirect sandwich ELISA assays on sera collected from each transplant subject before transplantation and at 1, 3, and 12 months after transplantation. Patients were excluded if they have history of diabetes, treatment with lipid-lowering medications or HIV protease inhibitors, prednisone dose > 15 mg/day, nephrotic range proteinuria, serum creatinine > 1.5 mg/dL, or active inflammatory disease. Sera from 18 transplanted patients were analyzed. Four subjects were excluded due to insufficient data. Twelve and eight patients had creatinine < 1.5 mg/dL at 3 and 12 months after transplantation, respectively. *Results*. Nitrated apoA-I was significantly reduced at 12 months after transplantation ($p = 0.039$). The decrease in apoA-I nitration was associated with significant reduction in myeloperoxidase (MPO) activity ($p = 0.047$). In contrast to apoA-I, nitrated apoB was not affected after kidney transplantation. *Conclusions*. Patients with well-functioning grafts had significant reduction in nitrated apoA-I 12 months after kidney transplantation. Further studies are needed in a large cohort to determine if nitrated apoA-I can be used as a valuable marker for cardiovascular risk stratification in chronic kidney disease.

1. Background

Cardiovascular disease (CVD) is the leading cause of death among patients with end-stage renal disease (ESRD), accounting for approximately half of all deaths [1–4]. The major course of treatment is dialysis. Dialysis patients die at six to seven times the rate of individuals in the general population with otherwise similar risk factors. Patients with less severe stages of chronic kidney disease (CKD) also carry excess risk of cardiovascular mortality that is not explained by traditional risk factors [5, 6]. Reduction of LDL cholesterol using statins lowers risk of atherosclerotic events in nondialysis CKD patients [7, 8]. However, similar protection by statins was not observed in clinical trials involving patients on dialysis [9, 10].

It is possible that HDL's quantity and quality may explain some of the residual risks in patients with advanced CKD. Epidemiologic studies have shown that there is a gradual increase in the risk of CVD as HDL cholesterol decreases [11, 12]. However, recent pharmacologic interventions aimed at increasing HDL abundance failed to provide clinical benefits and were associated with unexpected side effects despite promising preclinical outcomes [13, 14].

Recent studies are now focusing on assessing HDL function rather than measuring its traditional plasma concentration [15–17]. In this regard, attempts have been made to

measure nitrated apoA-I. These studies have advanced the concept that oxidative modification of HDL by MPO renders HDL dysfunctional [18–20]. Elevations in nitrated apoA-I containing HDL have been reported in atherosclerotic plaques and in plasma of CVD patients [6, 21–23].

Both qualitative and quantitative changes in HDL have been described in patients with CKD [24]. Specifically, HDL abundance is reduced and HDL acquires a proinflammatory property instead of its usual anti-inflammatory role [25, 26]. In addition, HDL from patients with CKD has reduced capacity for reverse cholesterol transport [27]. In more recent study, CKD has been shown to alter specific HDL functions linked to control of inflammation and endothelial responses [28]. Low HDL levels were associated with earlier entry in dialysis or doubling of plasma creatinine levels independently of the presence of diabetes [29]. Immunohistochemistry staining revealed higher nitrotyrosine in arteries with media calcification in CKD patients [30]. Moreover, plasma proteins of CKD patients showed a higher burden of nitration than that in healthy controls [31]. Despite these significant reports on plasma nitroproteome, the implication of nitrated lipoproteins in disease progression has not been explored in CKD patients.

Degree of nitration in apoA1-HDL has been evaluated by several methods such as mass spectrometry and Western blot analysis in different disease setting [23, 32, 33]. However, all these techniques are time-consuming and are not well adapted to high throughput screen setting. We have developed an ELISA based method to quantify serum nitrated apoA-I. We showed that CVD subjects have low total HDL but their nitrated apoA-I content is high [34]. Using the same ELISA, Vazquez et al. demonstrated a significant decrease in cholesterol efflux by ABCA1 transporters and impaired endothelial function that were associated with increased nitration of apoA-I-HDL in obese women [35]. Patients with CKD are at increased cardiovascular risk and have reduced HDL levels and altered HDL composition [36]. We have therefore investigated whether levels of circulating nitrated lipoproteins change in CKD patients treated with kidney transplantation.

2. Subjects and Methods

2.1. Patients Selection and Clinical Variable Assessment. Seventy-eight subjects were recruited from outpatient clinics of SUNY Downstate Medical Center between November 2010 and June 2013. The study was approved by the Institutional Review Board under protocol number 441318-1. Written informed consent was obtained from all study participants. All participants were adults. Kidney transplant recipients were enrolled in the study unless they met exclusion criteria. Patients with the following criteria were excluded from the study: diabetes, HIV on antiretroviral therapy, active systemic rheumatologic diseases, nephrotic syndrome, protein : creatinine ratio >1, treatment with lipid-lowering agents, body mass index (BMI) > 35, creatinine >1.5 mg/dL or estimated glomerular filtration (eGFR) <60 mL/min by Modification of Diet for Renal Disease (MDRD) equation, steroid dose >15 mg prednisone or equivalent a month or more after transplantation, and treatment with sirolimus or everolimus. All these above selection criteria would eliminate most of important confounding factors that could interfere with lipid and lipoprotein metabolism. A sample size of 12 was estimated to provide 90% power to detect difference of 448 μg/dL between pretransplant and 12-month posttransplant concentration of nitrated apoA-I at two-tailed type I error α of 0.05 using Wilcoxon signed-rank test [34, 35].

Sera were collected from each transplant subject before transplantation and at 1, 3, and 12 months after transplantation. In total, sera from eighteen transplanted patients who were eligible and presented stable kidney function were retrospectively analyzed. Four patients were excluded due to incomplete time point data. At the end, among this group, twelve and eight patients had creatinine < 1.5 mg/dL at 3 and 12 months after transplantation, respectively.

2.2. Sample Preparation and Quantification of Nitrated Lipoproteins by ELISA. Aliquoted sera stored at −70°C were thawed and concentrations of nitrated apoA1-containing HDL were measured using a well-established sandwich ELISA method [34]. A similar sandwich ELISA has been developed to measure levels of nitrated apoB-containing LDL particles as well. The specificity of the ELISA assay was described previously [34]. The intra-assay and interassay coefficients of variation for nitrated lipoproteins and apolipoproteins measurement were less than 5% and 10%, respectively. These values are consistent with the precision of typical sandwich ELISA assays [34, 37, 38]. Briefly, diluted sera samples were incubated in 96-well plates previously coated with monoclonal anti-nitrotyrosine antibodies (EMD Millipore, Billerica, Ma, USA; clone 1A6, Cat.# 05-233), enabling the capture of total nitrated serum proteins including apoA-I and apoB. The plates were blocked in PBS buffer containing 3% bovine serum albumin (BSA) and washed with PBS-Tween (0.05%). Primary polyclonal antibodies to human apoA-I or apoB (Novus Biologicals, Littleton, CO, USA; Cat.# NB400-147 and Cat.# NB120-7616, resp.) were added to specifically bind nitrated apoA-I or nitrated apoB captured by the anti-nitrotyrosine antibodies. Standard curves were generated using increasing concentrations (1–100 ng/mL and 1–100 μg/mL) of purified human serum HDL and LDL, respectively (MyBioSource, San Diego, CA, USA; Cat.# MBS173145 and Cat.# 173147). For this purpose, monoclonal antibodies against apoA-I and apoB were immobilized in 96-well plates (4H1 and 1D1, resp., University of Ottawa Institute, Ottawa, Canada). Bound HDL (apoA-I-HDL) and LDL (apoB-LDL) were detected with alkaline phosphatase-conjugated secondary antibodies and p-Nitrophenyl phosphate (pNPP) as substrate (1 mg/mL).

Absorbance at 405 nm was measured and corrected to absorbance of control wells in which PBS-Tween was added instead of serum. Total serum apoA-I and apoB levels were determined as described previously [34, 35]. Absolute values of nitrated apolipoproteins (apoA-I and apoB) were normalized to total serum apoproteins and degree of nitration was expressed as percent.

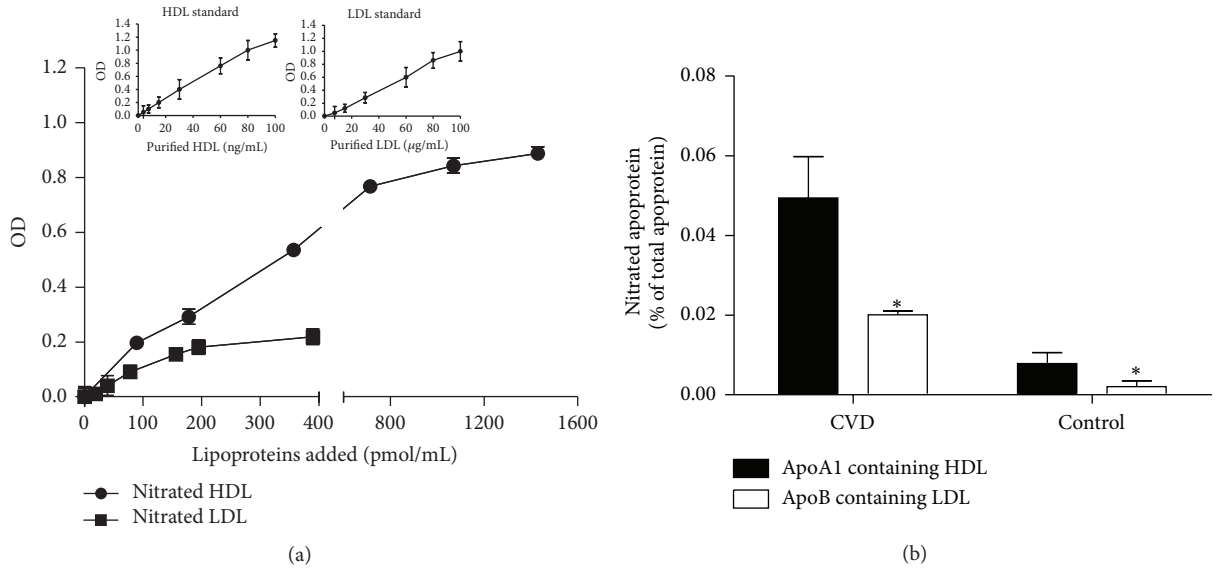

Figure 1: Panel (a) shows changes in optical densities due to the presence of nitrated apoA-I and apoB in the serum when immobilized anti-nitrotyrosine antibodies are incubated with increasing amounts of purified HDL and LDL. The amount of nitrated apolipoproteins present in the serum as measured by ELISA using commercially available purified human HDL and LDL as standard curves. Comparison between nitrated HDL (apoA-I-HDL) and nitrated LDL (apoB-HDL) was carried out using equimolar concentrations of HDL and LDL as described in methods. Representative linear standard curves for ELISA are plotted at the top of panel (a). Values are mean of triplicates ± SD. Panel (b) shows a comparison between percent nitration of apoA-I-HDL and apoB-LDL in human serum. Sera obtained from commercially available blood donors (Bioreclamation, LLC) were used. Mean values in these sera for apoB were 149.69 ± 28.78 mg/dL (range: 114.65–204.62 mg/dL) and for apoA-I were 49.30±12.96 mg/dL (range: 35.06–73.35 mg/dL). Sera were distributed in two groups (control versus CVD; $N = 10$/group) based on their lipids and lipoproteins levels. Concentrations of nitrated apoA-I and nitrated apoB and lipoproteins levels were measured by ELISA. Final values of nitrated apoA-I and apoB were normalized by levels of HDL and LDL, respectively. Values are mean ± SD ($N = 10$/group). One-way ANOVA test was performed between the two groups. Statistical significance was considered at $p < 0.05$.

2.3. Lipid Analyses and Enzymatic Assays. Non-HDL fraction was isolated from sera by precipitating (LDL/VLDL) with manganese chloride solution (1.06 M). After centrifugation, total cholesterol in the supernatants (HDLc) and in PBS-reconstituted precipitates (non-HDLc) was measured by colorimetric assay (Wako Diagnostics, Richmond, VA, USA; Cat.# 439-17501). Lipid peroxidation/oxidative stress were evaluated by measuring thiobarbituric acid reactive substances (TBARS) in sera. Briefly, the LDL/VLDL fraction was precipitated by 1.06 M manganese chloride and levels of TBARS were determined using OXI-TEK TBARS assay kit (ZeptoMetrix Corp., Buffalo, NY, USA; Cat.# 0801192). MPO was measured by colorimetric activity assay kit (Sigma, St. Louis, MO, USA; Cat.# MAK068).

2.4. Statistical Analysis. Only results for those with creatinine < 1.5 (those with successful transplant) are mentioned. Data for continuous variables (means ± SD) and medians (interquartile ranges) were reported. All analyses were performed using the Prism GraphPad 5.0 and Statistica 10.0 softwares. Paired values of percent nitrated apoA-I or nitrated apoB before and after transplantation were compared using nonparametric Wilcoxon signed-rank sum test. In addition, linear regression and Pearson's correlation coefficient were used to assess associations between variables. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Quantification of Nitrated HDL and LDL by ELISA. Figure 1(a) illustrates kinetic curve of detection of immobilized nitrotyrosine-bound lipoproteins in the wells by polyclonal antibodies against apoA-I and apoB. At equimolar concentrations, nitrated LDL binding reached saturation quicker than nitrated HDL, and the assay achieved linearity between 0 and 100 μg/mL LDL added and between 0 and 100 ng/mL HDL added (top 2 panels in Figure 1(a)). Sera from CVD patients showed a twofold increase of nitrated lipoproteins levels as compared to healthy subjects. There was approximately a sixfold increase of nitrated molecules of HDL compared to LDL (Figure 1(b)).

3.2. Clinical Characteristics of Study Cohort. Patients meeting eligibility criteria as described above were analyzed in this study. Among the 18 transplanted patients who were eligible and presented stable kidney function four patients were excluded due to incomplete data and twelve and eight patients had creatinine < 1.5 mg/dL at 3 and 12 months after transplantation, respectively.

Subjects' age in this cohort ranged from 29 to 64 years. Clinical characteristics are summarized in Table 1. At baseline, the mean HDL cholesterol and the mean non-HDL cholesterol were 52.9 ± 16.7 mg/dL (22.4–85.3) and

Table 1: Clinical characteristics at baseline (before transplantation) and at 3 months and 12 months after transplantation.

Characteristics	Baseline ($n = 14$)[a]	3 months after transplantation ($n = 12$)	12 months after transplantation ($n = 8$)
Age at transplantation (years)	46.6 ± 12	45.1 ± 11.9	48.3 ± 15.8
Female (n)	6	6	4
BMI (kg/m^2)	25.1 ± 3.26	24.7 ± 3.3	28.9 ± 4.1*
Systolic BP (mmHg)	149.5 ± 13.6	128.3 ± 14.7***	143.9 ± 18.1
Diastolic BP (mmHg)	86 ± 7.8	73.3 ± 11.8**	81.5 ± 10.8
Blood urea nitrogen, BUN (mg/dL)	39.8 ± 20.8	22.9 ± 5.1*	24.3 ± 8
Triglycerides (mg/dL)	121.30 [62.27–386.37]	135.60 [73.90–246.34]	103.40 [84.44–242.00]
Total cholesterol (mg/dL)	152.4 ± 36.5	187.3 ± 26.4*	198.3 ± 81.7
Glycerol (mg/dL)	28.5 ± 23.1	29.4 ± 11.6	39.1 ± 33.8
Serum apoA-I (mg/dL)	76.76 [9.68–92.61]	71.99 [48.43–90.33]	79.19 [45.04–89.19]
Serum apoB (mg/dL)	129.13 [74.29–186.95]	115.84 [59.21–254.67]	158.20 [61.96–190.96]
Non-HDL cholesterol (LDL + VLDL, mg/dL)	107.1 ± 22.2	97.6 ± 27.3	118.2 ± 18.6
HDL cholesterol (mg/dL)	52.9 ± 16.7	65.8 ± 30.4	52.6 ± 17.7
TBARS (LDL + VLDL) (MDA nmoles/mL)	7.7 ± 5.7	7.5 ± 6.3	6.9 ± 3.0
Myeloperoxidase (mU/mL)	109.7 ± 27.0	110.4 ± 50.1	73.9 ± 53.0*
Serum creatinine (mg/dL)	7.9 ± 2.5	1.2 ± 0.2***	1.2 ± 0.3***
eGFR (mL/min) (MDRD)	N/A	73.6 ± 14.5	74.9 ± 15.9
hs-CRP (mg/L) (median)[b]	<4	<4	<4
Hemoglobin (g/dL)	11.3 ± 2	12.5 ± 1.4	13.1 ± 1.4*
Albumin (g/dL)	4.1 ± 0.4	4.3 ± 0.2	4.1 ± 0.1

Data are presented as means ± SD or medians [interquartile ranges]. Values from baseline (before transplantation) and 3 months and 12 months (after transplantation) were compared using nonparametric test (data were significant at $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).
[a]Fourteen patients had creatinine ≤1.5 at 3 or 12 months after transplantation; two of the 14 patients were not included at 3 months because creatinine was >1.5. The graft function of these two patients improved to creatinine of ≤1.5, so, together with 6 patients included at 3 months, they make up 8 patients at 12 months.
[b]50% of patients had values <4 (lower limit of detection for assay = 4 mg/L).

107.1 ± 22.2 mg/dL (71.7–147.3), respectively. Mean value of triglycerides levels was 157.8 ± 89.6 mg/dL and apoA-I and apoB levels were 74.1 ± 12.4 mg/dL and 123.3 ± 29.8 mg/dL, respectively. Creatinine (Cr) levels ranged from 4.6 mg/dL to 13.2 mg/dL and C-reactive protein (CRP) levels were below 4 mg/L. There was a reduction in serum creatinine and there was a slight increase in BMI and hemoglobin levels 12 months after transplantation as compared to values at baseline.

3.3. Temporal Changes in Serum Components and Status of Nitrated Lipoproteins in the Total CKD Cohort. Overall, serum components such as total apoA-I and apoB did not change over time after transplantation (Figures 2(a) and 2(b)). Analysis of all fourteen patients with good graft function did not show any significant changes in nitrated lipoproteins 1 month, 3 months, and 12 months after transplantation (Figures 2(c)–2(f)).

3.4. Changes in Nitrated HDL and LDL in Kidney Transplant Recipients with Creatinine < 1.5 mg/dL. At the end of the study we had twelve patients and eight patients with good graft function (creatinine < 1.5 mg/dL) at 3 months and 12 months after transplantation, respectively. For each subject, we compared paired values of percent nitrated apoA-I and apoB before kidney transplantation and 3 months and 12 months after kidney transplantation. Analysis of the twelve patients that had creatinine < 1.5 mg/dL at 3 months after transplantation showed no difference in nitrated apoA-I (Figure 3(a)). At 12 months after transplantation, levels of apoA-I-HDL were slightly increased but did not reach significance (mean values were 73.4 ± 14.1 mg/dL and 76.7 ± 13.7 mg/dL at baseline and at 12 months after transplantation, resp.). This modest elevation of serum apoA-I levels was associated with significant reduction (~12%) in nitrated apoA-I (Figure 3(b)). Interestingly, six patients among eight have decreased their nitrated apoA-I by ~10%–30% 12 month after transplantation. The mean value for percent of nitrated apoA-I was significantly reduced by 18.5 ng/mg apoA-I (median value reduced by 22.5 ng/mg apoA-I; $p = 0.039$) 12 months after transplantation. In contrast, there were no significant changes in nitrated apoB at 3 months and 12 months after transplantation (Figures 3(c) and 3(d)). Like apoA-I, serum apoB levels tended to slightly increase but did not reach any significance (mean values were 112.46 ± 46.92 mg/dL and 141.65 ± 41.38 mg/dL at baseline and at 12 months after transplantation).

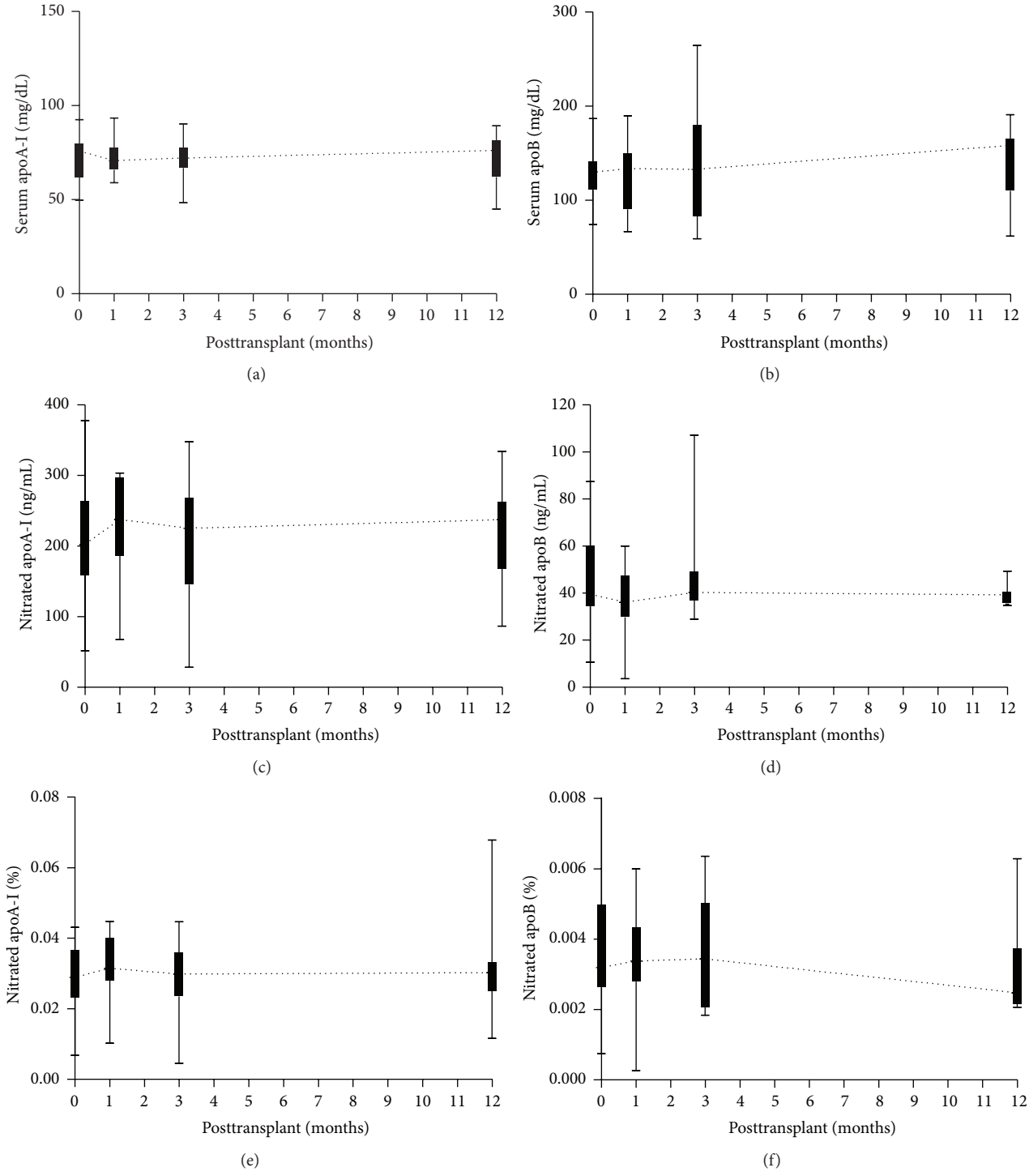

Figure 2: Temporal changes of circulating apolipoproteins and nitrated apolipoproteins in the 14 transplant patients with good kidney function before transplantation (baseline) and 1 month, 3 months, and 12 months after transplantation. Concentrations of total serum apoA-I containing HDL and apoB containing LDL particles (a and b) and levels of nitrated apoA-I and apoB (c and d) were measured by ELISA. Percentages of nitrated apoA-I and nitrated apoB were calculated by normalizing absolute values by total amount of apoA-I and apoB, respectively (e and f). Data are represented as box-and-whisker plots. Median values from each time point are connected to generate curves.

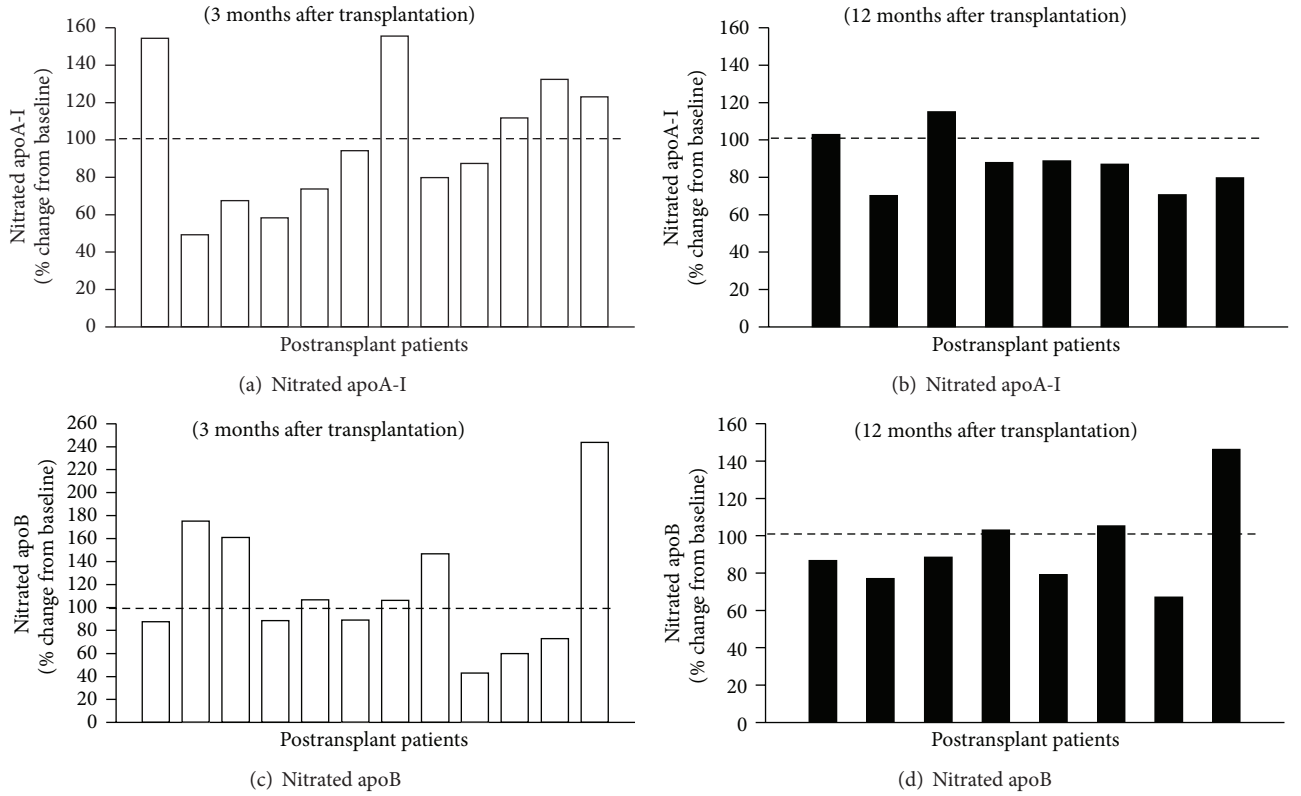

Figure 3: Percentage changes of nitrated lipoproteins (apoA-I-HDL and apoB-LDL) 3 months and 12 months after transplantation. Sera of twelve and eight patients with serum creatinine <1.5 mg/dL at baseline (before transplantation) and at 3 and 12 months after transplantation were analyzed by ELISA. Data represented are percent changes of nitrated apoA-I (a and b) and nitrated apoB levels (c and d) at 3 months and 12 months after transplantation, respectively. Baseline value for each patient was set at 100%. Paired values of percent nitrated apoA-I or nitrated apoB before and after transplantation were compared using nonparametric Wilcoxon signed-rank sum test. Statistical significance was considered at $p < 0.05$.

3.5. MPO Activity and Lipid Peroxidation. The decrease in apoA-I nitration 12 months after transplantation was associated with a significant reduction of MPO activity levels (Table 1; median values: 68.3 mU/mL versus 107.1 mU/mL; 32% decrease; $p = 0.047$). Lipid peroxidation as measured by TBARS assay was unchanged at 3 months and 12 months after transplantation.

3.6. Correlation between HDL and LDL Levels and Percent Nitrated apoA-I and Nitrated apoB. In our previous study, we have reported a negative relationship between degree of apoA-I-HDL and levels of circulating HDL particles in low HDL patients [34]. In this study, we sought to determine patterns of potential relationship between nitrated apolipoproteins and circulating lipoproteins in kidney transplant patients at baseline and at 3 months and 12 months after transplantation (Figure 4). There was no significant correlation between percent nitrated apoA-I and serum apoA-I levels in CKD patients before kidney transplantation (Figure 4(a)) and 3 months (Figure 4(b)) and 12 months (Figure 4(c)) after kidney transplantation. In contrast, there was a significant negative relationship between percent nitrated apoB and serum apoB levels at baseline (Figure 4(d)). This inverse correlation was maintained after 3 months (Figure 4(e)) and becomes markedly significant 12 months after kidney transplantation (Figure 4(f)). There was no significant association between nitrated apoA-I or nitrated apoB with other known inflammatory and cardiovascular markers such as hs-CRP, MPO, and Cr. (data not shown).

4. Discussion

CVD in CKD is primarily driven by oxidative stress, vascular calcification, hypertension, inflammation, and accumulation of oxidized lipoproteins as well as HDL deficiency and dysfunction [39, 40]. Recent study showed that HDL function was impaired in heart transplant recipients but it was not related to cardiac allograph vasculopathy and CRP levels [41]. To date, there is insufficient information about HDL functionality in kidney transplant recipients. In the present report, we demonstrate for the first time that serum nitrated apoA-I is reduced after 12 months in kidney transplant recipients with good kidney function.

We examined associations of nitrated lipoproteins with serum levels of apoA-I and apoB in kidney transplant recipients. We found no significant relationship between nitrated apoA-I and levels of circulating apoA-I at baseline and at 3

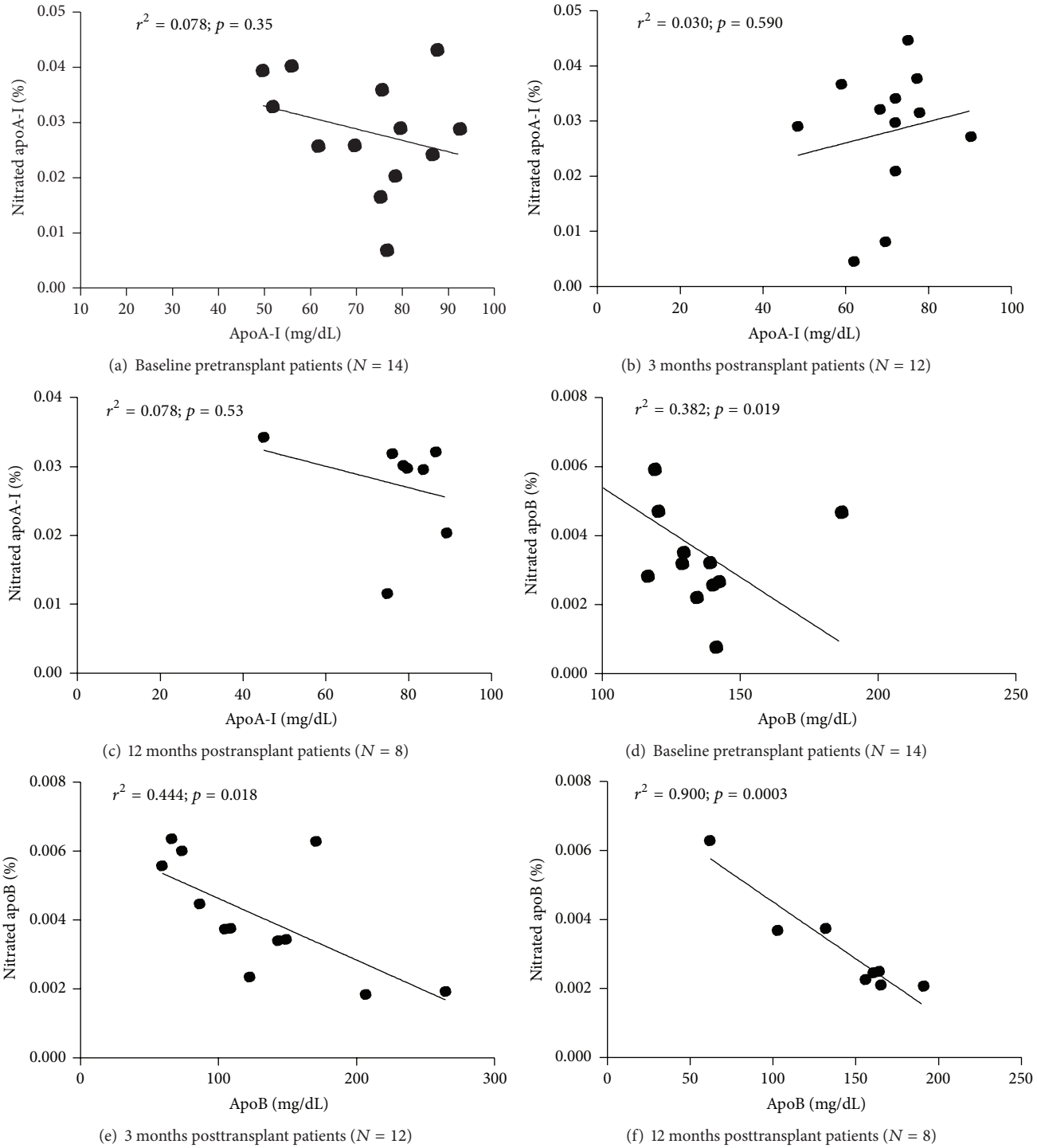

Figure 4: Correlations between % nitrated apoA-I and apoA-I-HDL levels and % nitrated apoB and apoB-LDL levels at baseline (before transplantation; a and d) and at 3 months (b and e) and 12 months (c and f) after transplantation, respectively. Linear regression was used to generate the curves. Statistical significance was considered at $p < 0.05$.

months and 12 months after transplantation (Figures 4(a)–4(c)). This is not in agreement with our previous observation in CVD patients with low HDL [34]. This could be due to possible differences in lipoproteins and/or degree of nitration and patient's population from CKD and CVD. Surprisingly, there was a significant negative correlation between percent nitrated apoB and levels of serum apoB at baseline and at 3 months and 12 months after transplantation (Figures 4(d)–4(f)). The more the serum is enriched with apoB the lesser the degree of apoB nitration is. One plausible explanation

to this unexpected observation could relate to differences in clearance of different forms of modified apoB-LDL particles. We speculate that nitrated apoB-LDL molecules would be cleared faster in the circulation than unmodified LDL resulting in lower amount of nitrated apoB. In fact, previous studies demonstrated that the accumulation of apoB-containing lipoproteins results primarily from decreased clearance rather than from increased synthesis [42]. LDL nitration has been shown to be associated with enhanced macrophages uptake when compared with oxidized LDL isolated from rheumatoid and osteoarthritis patients with CVD [43]. More studies are warranted to further elucidate the clearance of nitrated LDL particles by macrophages in CKD patients.

MPO is the main enzyme involved in chlorination and nitration of lipoproteins. Plasma MPO levels have been associated with coronary artery disease [44]. Cavusoglu et al. have shown that higher baseline MPO levels independently predict the occurrence of myocardial infraction within 2 years in patients with acute coronary syndrome [45]. High levels of nitrated HDL have been found in atherosclerotic lesions and plasma of CVD patients. In contrast, the majority of nitrated LDL mainly resides within atherosclerotic plaques [21, 46–48]. Use of drugs such as statins, beta-blockers, or ACE inhibitors has been shown to reduce MPO levels in patients with acute coronary syndrome but not in patients with stable coronary artery disease [49].

Interestingly, the decrease in apoA-I nitration 12 months after transplantation was associated with significant reduction of MPO activity (Table 1). It is not clear from our studies whether the reduced nitrated apolipoprotein A-I at 12 months is causally linked to reduced MPO activity. It is possible that reduction in serum concentration of MPO after successful kidney transplantation is due to attenuation of oxidant and inflammatory states induced by the uremia. In fact, recent study demonstrates that MPO deficiency ameliorates renal injury in the renal ablation model of CKD in mice [50]. Another study reports a negative correlation between MPO and urea and creatinine levels [51].

The evolution and severity of CKD have been shown to be associated with elevated oxidative stress [52]. Analysis of TBARS content in apoB-associated LDL/VLDL particles did not reveal any significant differences at 3 months and 12 months after transplantation compared to baseline (Table 1). This is quite different from the study by Vostálová et al. showing beneficial effect of successful kidney transplantation on the antioxidant status and lipid metabolism which resulted from both improved renal function and reduced cardiovascular complications [53]. One plausible explanation to this discrepancy could be related to differences in lipid status, patient selection, and protocol design between the two studies. In addition, the changes in lipid peroxidation products in CKD population are still debated [54–56].

In this study, a large proportion of subjects have high levels of serum apoB (>100 mg/dL; normal range ≤60 mg/dL) probably due to absence of lipid-lowering treatment in these patients and possible effect of immunosuppressive drugs. Unlike apoA-I, good kidney function did not affect degree of apoB nitration at 3 months and 12 months after transplantation (Figures 3(c) and 3(d)). These differences could be related to low levels of nitrated apoB in serum as compared to nitrated apoA-I. Alternatively, structural and molecular differences between lipoproteins may occur during progression of CKD, rendering modified LDL molecules in particular (oxidation, glycation, and carbamylation) less susceptible to nitration by MPO. Analysis of total population (all fourteen patients) with good graft function did not show any significant temporal changes in nitrated lipoproteins 1 month, 3 months, and 12 months after transplantation (Figure 2). This could be due to significant interindividual variations among patients in this small population. However, modest elevation of serum apoA-I levels was associated with significant reduction of nitrated apoA-I in kidney transplant recipients after only 12 months (Figure 3(b)). This is consistent with recent study reporting that changes of lipid profiles occur early and almost universally at 12 months after kidney transplantation [57].

One major limitation of our study is the small sample size due to strict patient's exclusion criteria setting. Nevertheless, none of our patients had received lipid-lowering therapy that could affect outcome of the study. Another limitation is lack of suitable normal control group (donors) for this particular setting protocol. These findings need to be confirmed in a large prospective study in order to validate the usefulness of nitrated apoA-I as independent predictor marker for CVD risk in CKD patients. More focused research is warranted to elucidate whether modified HDL in advanced CKD participates in very similar cellular processes of atherosclerosis such as foam cell formation, proliferation and migration of smooth muscle cells, and, most importantly, plaque destabilization.

5. Conclusion

In summary, we have demonstrated that patients with well-functioning kidney transplants had significant reduction in nitrated apoA-I-HDL 12 months after kidney transplantation without any major changes in nitrated apoB-LDL. Given the high cardiovascular burden of kidney transplant recipients, nitrated apoA-I may serve as valuable marker for population stratification and perhaps as a possible target for novel therapeutic strategies.

Abbreviations

ABCA1: ATP-binding cassette transporter A1
ApoA-I: Apolipoprotein A-I
ApoB: Apolipoprotein B
BUN: Blood urea nitrogen
CKD: Chronic Kidney disease
Cr.: Creatinine
CRP: C-reactive protein
CVD: Cardiovascular disease
EGFR: Estimated glomerular filtration
ELISA: Enzyme linked immunosorbent assay
ESRD: End-stage renal disease
HDL: High-density lipoprotein
LDL: Low-density lipoprotein
MDA: Malondialdehyde
MDRD: Modification of diet in renal disease

MPO: Myeloperoxidase
VLDL: Very low-density lipoprotein
TBARS: Thiobarbituric acid reactive substances.

Disclaimer

All authors agree to be accountable for all aspects of this work. Each author has participated sufficiently in this work and takes public responsibility for appropriate portions of the content.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Ahmed Bakillah performed experiments and participated in the acquisition of data and interpretation of results, wrote and revised the paper, and has given approval for the final paper. Fasika Tedla was responsible for the conception and design of the study and revising the paper and has given approval for the final paper. Isabelle Ayoub was responsible for the study design and samples collection and has given approval for the final paper. Devon John and Allen J. Norin revised the paper and have given approval for the final paper. M. Mahmood Hussain was responsible for the interpretation of the data and paper editing in a critical fashion and has given approval for the final paper. Clinton Brown was responsible for the conception and design of the study and revising the paper and has given approval for the final paper. All authors read and approved the final paper.

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Research Article

Circulating Irisin Levels Are Not Regulated by Nutritional Status, Obesity, or Leptin Levels in Rodents

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Irisin is a cleaved and secreted fragment of fibronectin type III domain containing 5 (FNDC5) that is mainly released by skeletal muscle and was proposed to mediate the beneficial effects of exercise on metabolism. In the present study we aim to investigate the regulation of the circulating levels of irisin in obese animal models (diet-induced obese (DIO) rats and leptin-deficient (ob/ob) mice), as well as the influence of nutritional status and leptin. Irisin levels were measured by Enzyme-Linked Immunosorbent Assay (ELISA) and Radioimmunoassay (RIA). Serum irisin levels remained unaltered in DIO rats and ob/ob mice. Moreover, its circulating levels were also unaffected by fasting, leptin deficiency, and exogenous leptin administration in rodents. In spite of these negative results we find a negative correlation between irisin and insulin in DIO animals and a positive correlation between irisin and glucose under short-term changes in nutritional status. Our findings indicate that serum irisin levels are not modulated by different physiological settings associated to alterations in energy homeostasis. These results suggest that in rodents circulating levels of irisin are not involved in the pathophysiology of obesity and could be unrelated to metabolic status; however, further studies should clarify its precise role in states of glucose homeostasis imbalance.

1. Introduction

The combination of energy-dense diets and sedentary lifestyles has accelerated the incidence of obesity and its comorbidities including dyslipidemia, diabetes mellitus, and cardiovascular disease. Inflammatory processes play a crucial role in the development of obesity. Adipose tissue release cytokines such as IL-6 and IL-1β in obesity that targets several tissues such as the heart, the pancreas, or the liver [1]. Physical inactivity causes accumulation of visceral fat that induces systemic low grade inflammation and in turn exercise promotes a variety of metabolically beneficial effects in the organism [2]. However, its mechanism of action remains elusive and many efforts are focused to characterize new molecular targets or mediators of these healthy benefits. The skeletal muscle is considered nowadays as a complete endocrine organ [3] and there are more than 1000 genes activated by exercise [4]. One of these proposed mediators is the peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1-α), a transcriptional coactivator released by the muscle that induces mitochondrial biogenesis and thus thermogenesis [5, 6] as well as modulating glucose, lipid, and energy homeostasis [7, 8]. PGC1-α induces the expression of the fibronectin type III domain containing 5 (FNDC5) gene that is cleaved and secretes a new hormone from skeletal muscle named irisin [8]. FNDC5 was simultaneously characterized in 2002 by two independent groups [9, 10], but it was ten years later when irisin attracted more interest because it was reported to act as a mediator of the beneficial effect of exercise [8], increasing metabolic

uncoupling and caloric expenditure and promoting browning [8, 11, 12], resulting in an improvement of obesity and glucose homeostasis. However, further studies performed in animals and humans reported controversial results and the physiological role of irisin is still under debate [2, 8, 13–18].

On the other hand, several studies assessed circulating levels of irisin in humans and found controversial results because some of them reported an increase of irisin under conditions of obesity [19, 20] while others found a negative association between irisin levels and obesity [21]. Furthermore recently no association was found between these two parameters in humans [22]. Accordingly, circulating irisin levels decreased after surgical [23] or dietary-induced [24] weight loss. In rodents, gene expression results showed that skeletal muscle FNDC5 mRNA levels were proportional to circulating leptin levels [25] and the regulation of FNDC5 expression in DIO animals was different depending on the fat depot [26]. However, there are no data on circulating irisin levels in obese rodents and its regulation under certain physiological conditions associated to body weight changes is very scarce. The aim of this study was to assess the regulation of serum irisin levels by nutritional status, leptin, and diet-induced obesity in rodents.

2. Material and Methods

2.1. Animals. Male Sprague-Dawley rats (bred in the Animalario Xeral of USC; Santiago de Compostela, Spain) and ob/ob mice (Charles River, Barcelona, Spain) were housed under conditions of controlled illumination (12 : 12-h light/dark cycle), humidity, and temperature. Animals were fed with a standard diet (Scientific Animal Food & Engineering, proteins 16%, carbohydrates 60%, and fat 3%) and tap water *ad libitum* unless otherwise indicated. The animals were sacrificed in a room separate from other experimental animals, and blood samples were centrifuged at 1500 g for 10 min and the serum was harvested and stored at −80°C until an analysis could be performed. All experimental procedures were reviewed and approved by the Ethics Committees of the University of Santiago de Compostela in accordance with the institutional guidelines and in strict compliance with the European Union normative for the care and use of experimental animals. The number of animals used in each experimental setting is indicated in the corresponding figure's caption.

2.2. Experimental Setting 1: Effect of the Diet on Serum Irisin Levels. After weaning, 6-week-old male Sprague-Dawley rats were either fed a high-fat (HF) diet (60% by energy) or a low-fat (LF) diet (10% by energy) (reference #: D12492 and D12450B resp., Research Diets, NJ, US) for 10 weeks.

2.3. Experimental Setting 2: Effects of Food Deprivation on Serum Irisin Levels. 10-week-old male rats were deprived of food for 48 h, or refed for 24 h after fasting while the control group was fed *ad libitum* [27, 28]. All animals had free access to tap water.

2.4. Experimental Setting 3: Effects of Leptin on Serum Irisin Levels. The effects of systemic leptin administration on serum irisin levels were studied in leptin deficient mice (ob/ob mice). Leptin-deficient animals were referred to wild type (WT) control animals and were distributed in three groups: (a) i.p. vehicle fed *ad libitum*, (b) i.p. vehicle after 36 h fasting, and (c) i.p. leptin in fed *ad libitum* mice. Animals were treated with recombinant leptin (L-4146, Sigma-Aldrich) at a dose of 0.5 mg/kg of body weight every 6 h for 3 days (intraperitoneal injection) [29].

2.5. Measurement of Body Composition. Body composition (fat and lean mass) was assessed using Nuclear Magnetic Resonance imaging system (Whole Body Composition Analyzer; EchoMRI, Houston, USA) as previously shown [30, 31].

2.6. Measurement of Biochemical and Hormonal Parameters. Irisin and insulin levels were determined by ELISA using reagents kits and methods provided by Phoenix Pharmaceuticals Inc. and by Millipore corporation, respectively. These kits are suitable for human, rats, and mice and were used in previous studies [20, 24, 32–34]. The quantitative measurement of irisin in serum samples was performed using a commercial ELISA kit directed against amino acids 33–142 of the FNDC5 protein (Irisin ELISA Kit EK-067-52; Phoenix Pharmaceuticals Inc., CA) according to the manufacturer's instructions. The absorbance from each sample was measured in duplicate using a spectrophotometric microplate reader at wavelength of 450 nm (Versamax Microplate Reader; Associates of Cape Cod Incorporated, East Falmouth, MA). The intra- and interassay coefficients of variation for the kit were 4–6% and 8–10%, respectively. Irisin levels were determined also by RIA using reagents kits and methods provided by Phoenix Pharmaceuticals Inc. (Belmont, CA; Cat. No. RK-067-16). For testing serum irisin levels, the samples were obtained from trunk blood by decapitation and were collected in tubes for serum separation (BD Vacutainer SST II Advance). Results were expressed as ng per milliliter of irisin in serum. Serum samples were tested in duplicate within one assay, and the results were expressed in terms of the rat irisin standard (ng/mL).

The absorbance from each sample with regard to insulin levels was measured in duplicate using a spectrophotometric microplate reader at wavelength of 450 nm and 590 nm. The intra- and interassay coefficients of variation for the kit were 1–4% and 6–9%, respectively. Serum samples were tested in duplicate within one assay, and the results were expressed in terms of the rat insulin standard (ng/mL).

Glucose and total cholesterol was determined using colorimetric assays (Spinreact, Girona, Spain) as previously described [35].

2.7. Western Blot Analysis. Western blot was performed as previously described [36, 37]. White adipose tissue (WAT) and brown adipose tissue (BAT) were dissected and stored at −80°C until further processing.

WAT and BAT were homogenized in ice-cold lysis buffer containing 50 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EGTA, 1 mmol/L EDTA, 1% Triton X-100, 1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride, 5 mmol/L sodium pyrophosphate, 0.27 mol/L sucrose, 0.1% 2-mercaptoethanol,

Table 1: Body composition and biochemical and hormonal characteristics from the models studied.

Group	Insulin (ng/mL)	Glucose (mg/dL)	Total cholesterol (mg/dL)	Fat mass (g)	Lean mass (g)
Low fat	5.36 ± 1.08	18.51 ± 1.02	6.32 ± 0.40	56.53 ± 5.63	412.3 ± 4.0
High fat	9.07 ± 1.58*	25.86 ± 0.96***	7.14 ± 0.92	150.02 ± 23.04**	435.1 ± 5.5**
Wild type mice	2.01 ± 0.42	2.41 ± 0.13	0.78 ± 0.07	—	—
ob/ob mice FED	7.67 ± 1.63**	4.40 ± 0.72*	1.04 ± 0.03*	—	—
ob/ob mice FAST	4.35 ± 0.85	1.94 ± 0.34$^{\#\#}$	0.088 ± 0.04	—	—
ob/ob mice FED + OB	1.32 ± 0.97$^{\#\#\#}$	3.24 ± 0.56	0.087 ± 0.02	—	—
Rats FED	1.11 ± 0.22	20.16 ± 2.09	5.09 ± 0.83	26.21 ± 0.88	175.6 ± 3.4
Rats FAST	0.09 ± 0.007**	13.23 ± 0.71**	8.20 ± 0.32**	21.94 ± 0.71*	163.5 ± 1.9*
Rats REFED	1.13 ± 0.19$^{\#\#}$	26.12 ± 0.78$^{*/\#\#\#}$	6.14 ± 0.34$^{\#}$	23.52 ± 1.13	166.6 ± 3.4

Values are mean ± SEM of 7-8 animals per group. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus controls. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ versus ob/ob FED and versus FAST 48 h.

and complete protease and phosphatase inhibitor cocktail (1 tablet/50 mL; Roche Diagnostics, Mannheim, Germany). Homogenates were centrifuged at 13,000 g for 10 min at 4°C, supernatants were removed, and aliquots were stored in −80°C.

WAT and BAT lysates were subjected to SDS-PAGE gels. Briefly, total protein lysates from WAT and BAT (20 μg) were subjected on 8% and 12% of SDS polyacrylamide gels and electrotransferred on a polyvinylidene difluoride membrane. Membranes were blocked for 1 h in TBS-Tween 20 (TBST: 50 mmol/L Tris-HCl [pH 7.5], 0.15 mol/L NaCl, and 0.1% Tween 20) containing 3% of BSA and probed with for 16 h at 4°C in TBST, 3% BSA in the indicated antibodies: IL-6 (ab-6672), IL-1β (ab-9722) (Abcam, Cambridge, UK); PGC1α (sc-13067) (Santa Cruz Biotechnology, Santa Cruz, CA), α-tubulin (T-5168), and β-actin (A-5316) (Sigma-Aldrich). For protein detection we used horseradish peroxidase-conjugated secondary antibodies (Daku Denmark, Glostrup, Denmark) and chemiluminescence (Pierce ECL Western Blotting Substrate, Thermo scientific, USA). Then, the membranes were exposed to X-ray film (Super RX, Fuji Medical X-Ray Film, Fujifilm, Japan) and developed with developer and fixing liquids (AGFA, Germany) under appropriate dark room conditions. We used seven samples per group and the protein levels were normalized to β-actin (WAT) and to α-tubulin (BAT) for each sample.

2.8. Statistics. The results are shown as the mean ± standard error of mean. Statistical analysis was performed using Student's t-test (when two groups were analysed) or one-way ANOVA followed by a *post hoc* multiple comparison test (Tukey Test) (when more than two groups were analysed). A P value less than 0.05 was considered statistically significant; Graph Prism software (San Diego, CA) was used for the data analysis. For correlation studies statistical analyses were performed using SPSS version 20.0 software statistical package (SPSS, Chicago, IL). The relationships between variables were analyzed by Pearson's correlation (normally distributed data) or Spearman's rank correlation (nonnormally distributed data) coefficients (r). A P value less than 0.05 was considered statistically significant.

3. Results

As expected, the higher body weight in rats fed HF diet (Figure 1(a)) was consistent with a significant increase in fat and lean mass index compared to LF diet fed rats (Table 1) and a significant increase in proteins levels of IL-1β in WAT but not of IL-6 (Figure 2(a)). We do not have a clear explanation for this fact but it could be due to the limited time of exposition to the diet (10 weeks) or because a total positive correlation between IL-6 and obesity is not always a strong proof, due to the fact that a lack of IL-6 has been shown to cause obesity and insulin resistance in rodents [38]. However, the DIO rats eat less amount of food than the LF diet fed rats because the HF diet is a hypercaloric food and these rats need less quantity of food to be satiated compared to hypocaloric LF diet fed animals (Figure 1(e)). Correspondingly, these DIO rats are hyperglycemic and hyperinsulinemic (Table 1) and display a trend of high cholesterol levels although this trend is not significant (Table 1), as previously described [22]. With regard to the monogenic model of obesity, the ob/ob mice exhibit a high body weight (Figure 1(b)) and food intake (Figure 1(f)), and similar to the DIO rats, the ob/ob mice display a significant increase in insulin, glucose, and cholesterol levels with respect to WT controls (Table 1) and high levels of inflammatory markers in WAT (Figure 2(b)). Furthermore, we measure the levels of these biochemical and hormonal parameters in the serum of rats submitted to short-term changes in nutritional status. As expected, a 48 h fasting induces a significant decrease in glucose and insulin serum levels compared to *ad libitum* fed animals and a significant increase in total cholesterol levels (Table 1) [39, 40].

Since circulating irisin levels are increased in human obesity [19, 20], we assessed serum irisin levels in two animal obese models: DIO rats as a model of polygenic obesity and ob/ob mice as a model of monogenic obesity. In both rodent obese models, serum irisin levels remained unaltered. Rats fed a HF diet showed similar serum irisin levels as animals fed a LF diet during 10 weeks (Figure 3(a)). Interestingly, these results are consistent with a recent study in humans [22]. Similarly, circulating irisin levels remained unaltered between WT and obese mice lacking leptin (ob/ob mice)

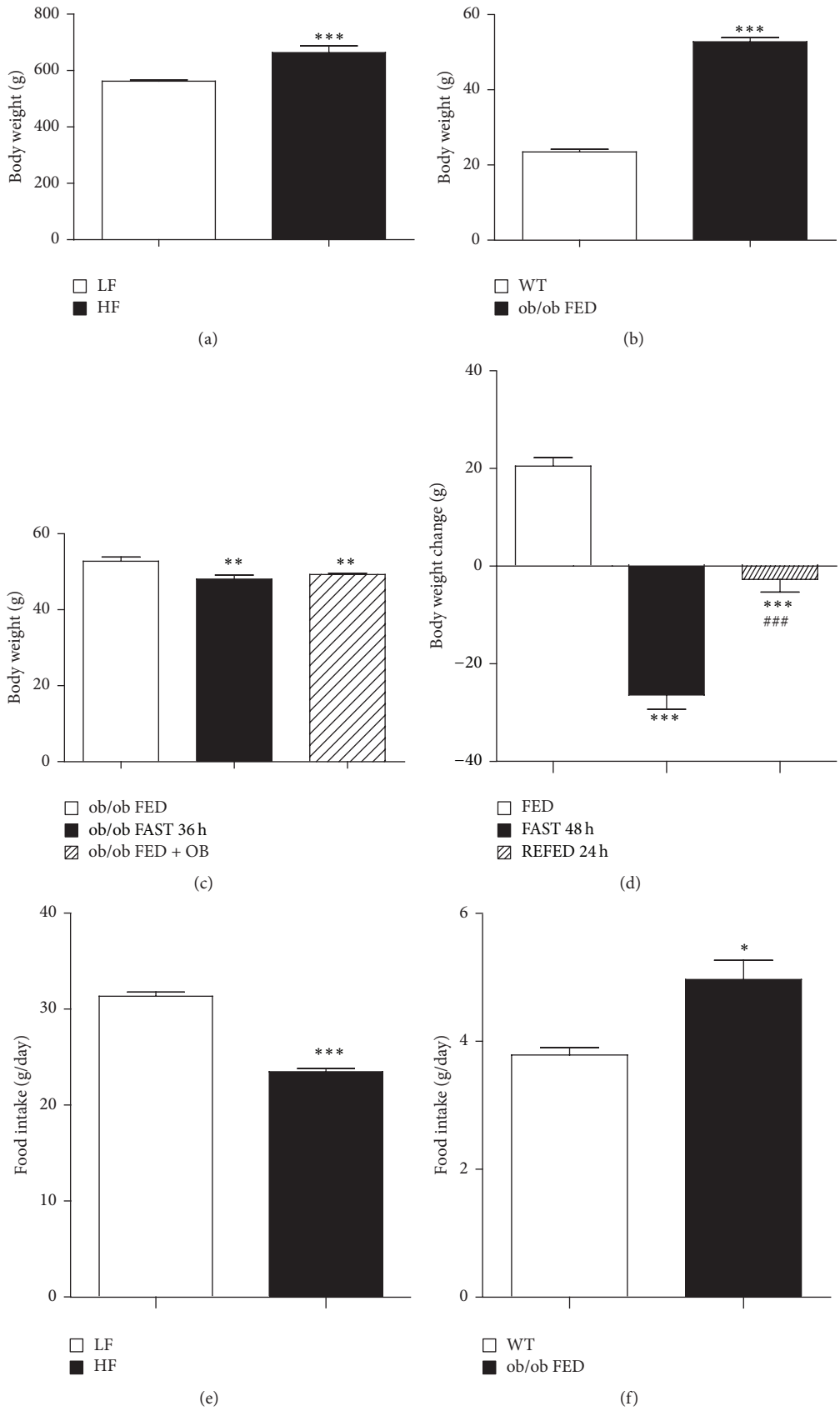

Figure 1: Continued.

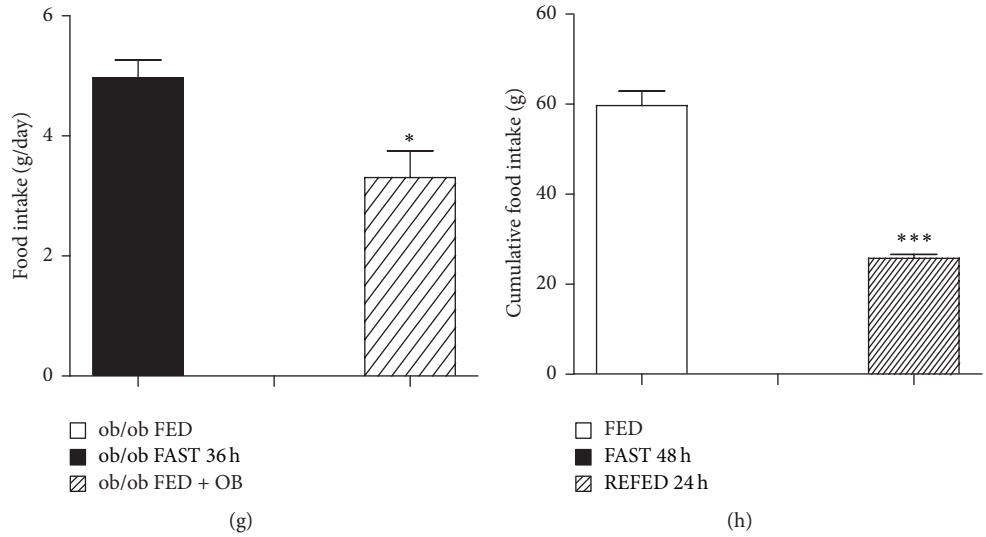

Figure 1: Body weight and food intake. (a) Body weight of rats fed with high fat (HF) and low fat (LF) diet. (b) Body weight in leptin deficient mice (ob/ob) versus wild type (WT) mice fed *ad libitum*. (c) Body weight in ob/ob mice fed *ad libitum*, 36 hours fasted and after leptin treatment in ob/ob mice fed *ad libitum*. (d) Body weight change in rats fed *ad libitum*, in 48 h of fasting and in rats submitted to a refeeding for 24 h after 48 h of fasting. (e) Food intake of rats fed with high fat (HF) and low fat (LF) diet. (f) Food intake in leptin deficient mice (ob/ob) versus wild type (WT) mice fed *ad libitum*. (g) Food intake in ob/ob mice fed *ad libitum*, 36 hours fasted and after leptin treatment in ob/ob mice fed *ad libitum*. (h) Food intake in rats fed *ad libitum*, in 48 h of fasting and in rats submitted to a refeeding for 24 h after 48 h of fasting. Values are mean ± standard error of the mean of 6–12 animals per group. Values are mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus controls and $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ versus FAST 48 h.

(Figure 3(b)). Of note, lack of differences in circulating irisin in both models of obesity was found in spite of different body weights (Figures 1(a) and 1(b)). As irisin levels were unchanged in animal models of long-term obesity, we next aimed to determine whether irisin might be regulated by short-term changes in nutritional status. In order to test this hypothesis, we measured irisin in animals fed *ad libitum* and after food deprivation. Serum irisin levels remained unaltered after 48 h of fasting or when rats were refed for 24 h (Figure 3(c)). As leptin is one of the main players in the regulation of energy balance, we carried out a food deprivation and leptin replacement in ob/ob mice to corroborate our previous data. Additionally, we measured the levels of PGC1-α, a proposed upstream regulator of irisin in BAT. We found a very pronounced decreased in protein levels of PGC1-α in ob/ob mice compared to WT mice (Figure 2(c)); however, these levels were increased by leptin treatment (Figure 2(c)) as previously described [41, 42].

In agreement with our previous findings, we failed to detect significant changes in circulating irisin levels in ob/ob mice fasted for 36 h in comparison to ob/ob mice fed *ad libitum* (Figure 3(d)), and the administration of exogenous leptin to ob/ob mice did not cause any significant alteration in serum irisin levels (Figure 3(d)). However, even though the food deprivation and leptin caused a marked decrease in the body weight (Figure 1(c)) and food intake (Figure 1(g)) and changes in proteins levels of PGC1-α (Figure 2(c)) of leptin-deficient mice.

Of note, this lack of differences in serum irisin levels in all of these animal models was replicated in an independent experiment by using a Radioimmunoassay (data not shown).

Finally, we have also performed correlations studies between serum irisin versus body composition parameters and biochemical and hormonal levels (Figures 4 and 5; Table 2). We do not find any correlation between fat mass, lean mass, or cholesterol levels and irisin in the different models studied (Figures 4(a), 4(b), 4(c), and 4(d); Figures 5(a), 5(c), 5(d), 5(e), 5(f), 5(h), and 5(i); Table 2); however, we detect a negative correlation between irisin and insulin in the DIO model (Figure 5(b); Table 2) as previously described in humans [21] and a positive correlation between glucose and irisin under short-term changes in nutritional status (Figure 5(g); Table 2).

4. Discussion

The discovery of irisin has created a great expectation due to its proposed beneficial metabolic effects. However, the knowledge about irisin regulation and secretion is still scant and the results are controversial. In this sense, the levels of circulating irisin in obesity have been reported to be negatively associated to obese men [21], whereas others indicated that irisin levels are high in obese individuals or are positively correlated to body mass index [19, 20, 23, 43]. In rodents, the results are equally ambiguous, as it was shown that serum irisin levels are decreased in obese Otsuka Long-Evans Tokushima Fatty (OLETF) rats in comparison to lean rats [25], and in obese Zucker rats [26] but increased in DIO rats [26]. In the current study, we have analyzed serum irisin levels in two different obese animal models, DIO rats as a model of polygenic obesity and in ob/ob mice as a model of monogenic obesity. In both cases, our results were identical,

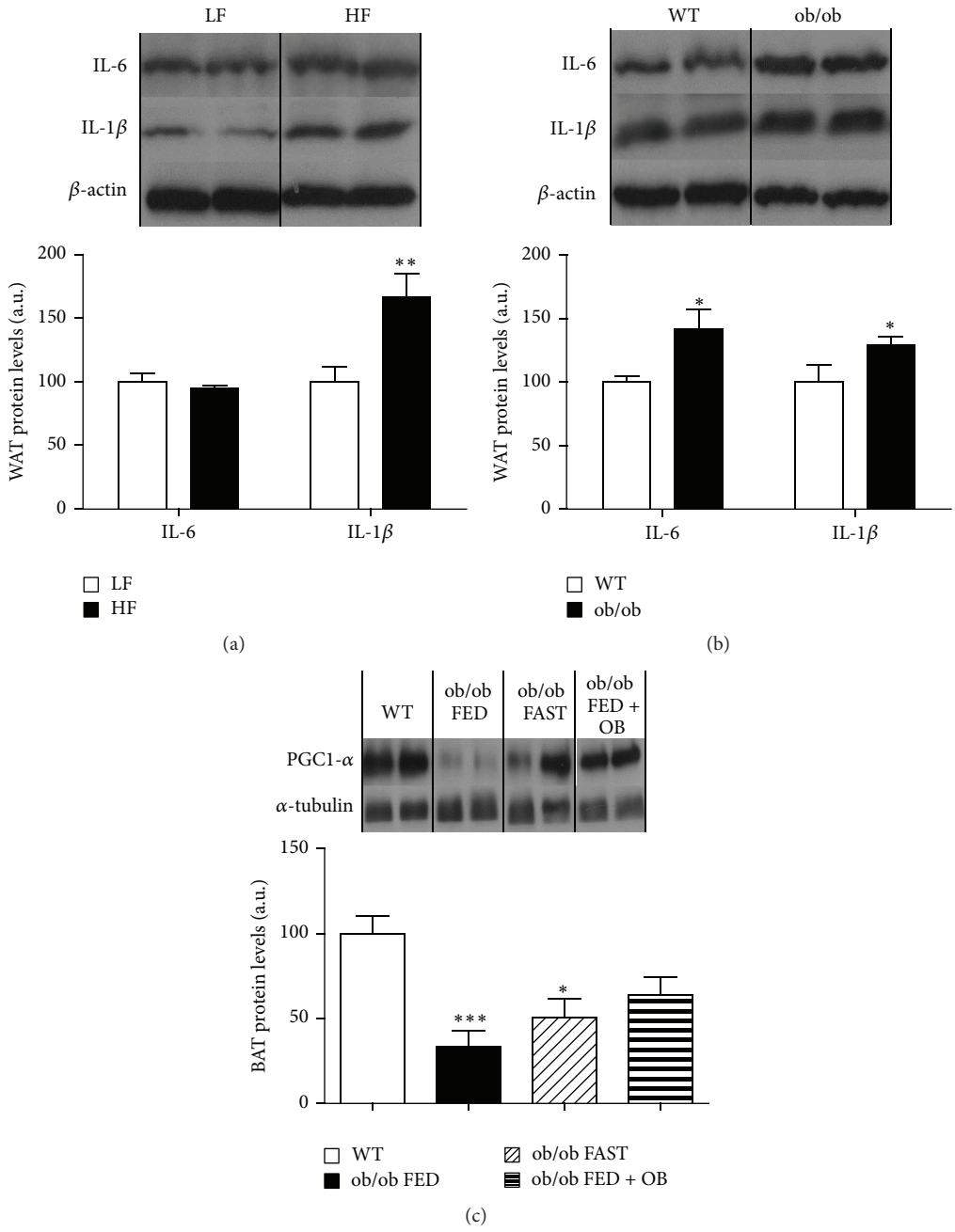

Figure 2: Measurements of protein levels in white and brown adipose tissue. WAT protein levels of IL-6 and IL-1β in DIO animals (a) and in ob/ob mice (b). BAT protein levels of PGC1-α in ob/ob mice (c). β-actin for WAT and α-tubulin for BAT were used to normalize protein levels. Values are mean ± SEM of 7-8 animals per group. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus controls.

as we did not detect any significant difference between lean and obese animals in concordance with a study in obese humans [22]. Although all these results obtained in different models of obese animals do not point in the same direction, it is important to highlight that each model is associated with different metabolic alterations that might affect irisin levels. For instance, OLETF rats are diabetic whereas DIO rats are insulin resistant but not diabetic. This is a key issue since the role of irisin on glucose metabolism is still under debate [21, 32, 44, 45]. Another crucial aspect that requires attention is the methodology used for the measurement of irisin. These methodological aspects are likely affecting the results in DIO rats. In this sense, the study of Roca-Rivada and colleagues assessed serum irisin levels by Western blot [26] whereas we used a radioimmunoassay-based method. In spite of these discrepancies between different animal models or methodologies, our present results suggest that circulating irisin levels are not affected by obesity in rodents.

Since obese rodents represent models with long-term metabolic alterations and compensatory mechanisms, we

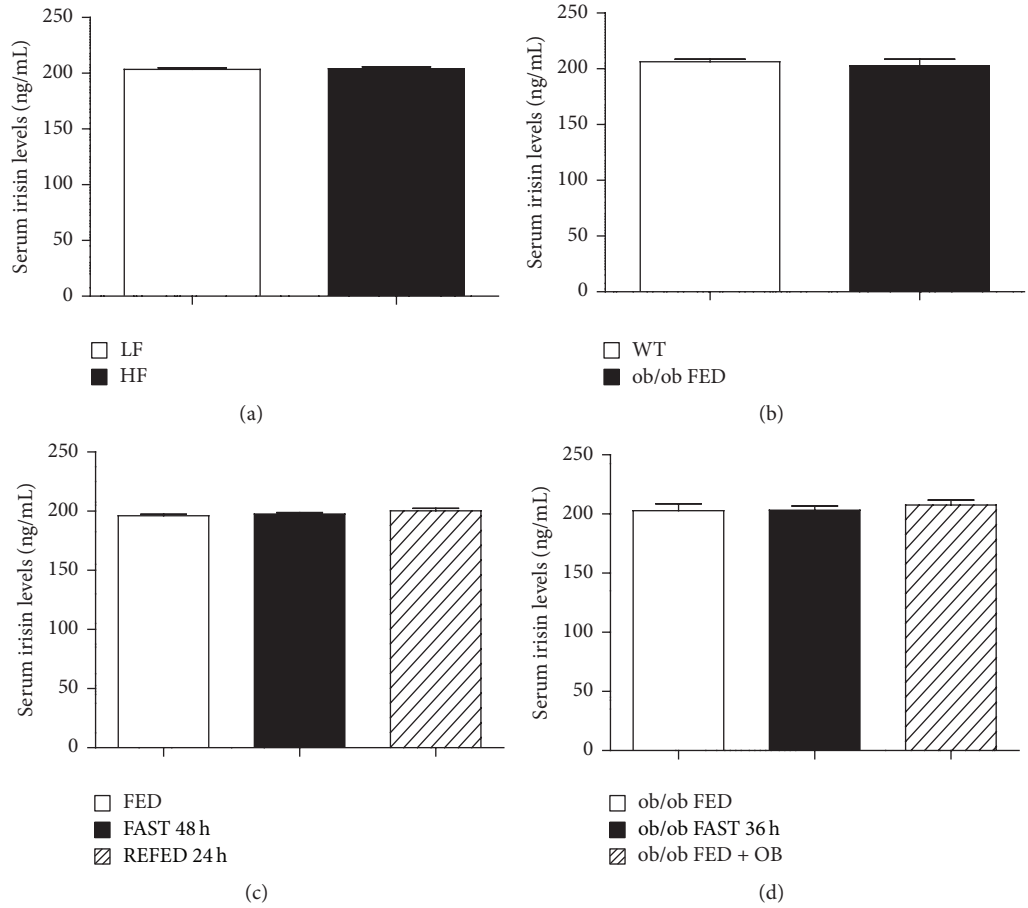

Figure 3: Serum irisin levels. (a) Serum irisin levels in rats fed with high fat (HF) and low fat (LF) diet for 10 weeks (b) Serum irisin levels in leptin deficient mice (ob/ob) versus wild type (WT) mice fed *ad libitum*. (c) Serum irisin levels in animals fed *ad libitum* (FED), fasted for 48 hours (FAST 48 h) or refed for 24 hours (REFED 24 h) after 48 hours of fasting. (d) Serum irisin levels in ob/ob mice fed *ad libitum*, 36 hours fasted, and in ob/ob mice fed *ad libitum* after leptin treatment. Values are mean ± SEM of the mean of 6–12 animals per group. A $P < 0.05$ was considered significant.

Table 2: Correlations of irisin with body composition and biochemical and hormonal parameters from the models studied.

Group	Irisin				
	Insulin (ng/mL)	Glucose (mg/dL)	Total cholesterol (mg/dL)	Fat mass (g)	Lean mass (g)
DIO	$r = -0{,}668$	$r = -0{,}213$	$r = 0{,}288$	$r = -0{,}213$	$r = -0{,}213$
	$P = 0.005^{**}$	$P = 0.446$	$P = 0.299$	$P = 0.446$	$P = 0.446$
ob/ob mice	$r = 0{,}045$	$r = 0{,}06$	$r = -0{,}229$	—	—
	$P = 0.818$	$P = 0.974$	$P = 0.242$	—	—
FED-FAST-REFED	$r = 0{,}250$	$r = 0{,}432$	$r = -0{,}123$	$r = -0{,}250$	$r = 0{,}040$
	$P = 0.262$	$P = 0.045^{*}$	$P = 0.587$	$P = 0.261$	$P = 0.859$

Statistical significance is from Pearson (normally distributed data) and from Spearman (nonnormally distributed data) correlation test.

next evaluated if irisin levels might be affected in situations of metabolic alterations at short term. The nutritional status induces changes in a variety of endocrine axis and if irisin has been proposed as a metabolic regulator, we hypothesized that it should be regulated by fasting. However, we failed to find any change in serum irisin levels by caloric restriction or refeeding, indicating that its circulating levels are not affected by nutritional status. Our findings are in agreement with a previous study indicating that weight loss induced by caloric restriction did not regulate circulating irisin levels in rats [46]. On the other hand both fasting and caloric restrictions are associated to a number of metabolic alterations, and one of the most relevant is the decreased leptin levels. Previous works have suggested a possible cross talk between leptin and irisin because irisin levels are associated with leptin in humans and rats [21, 25], and leptin increases

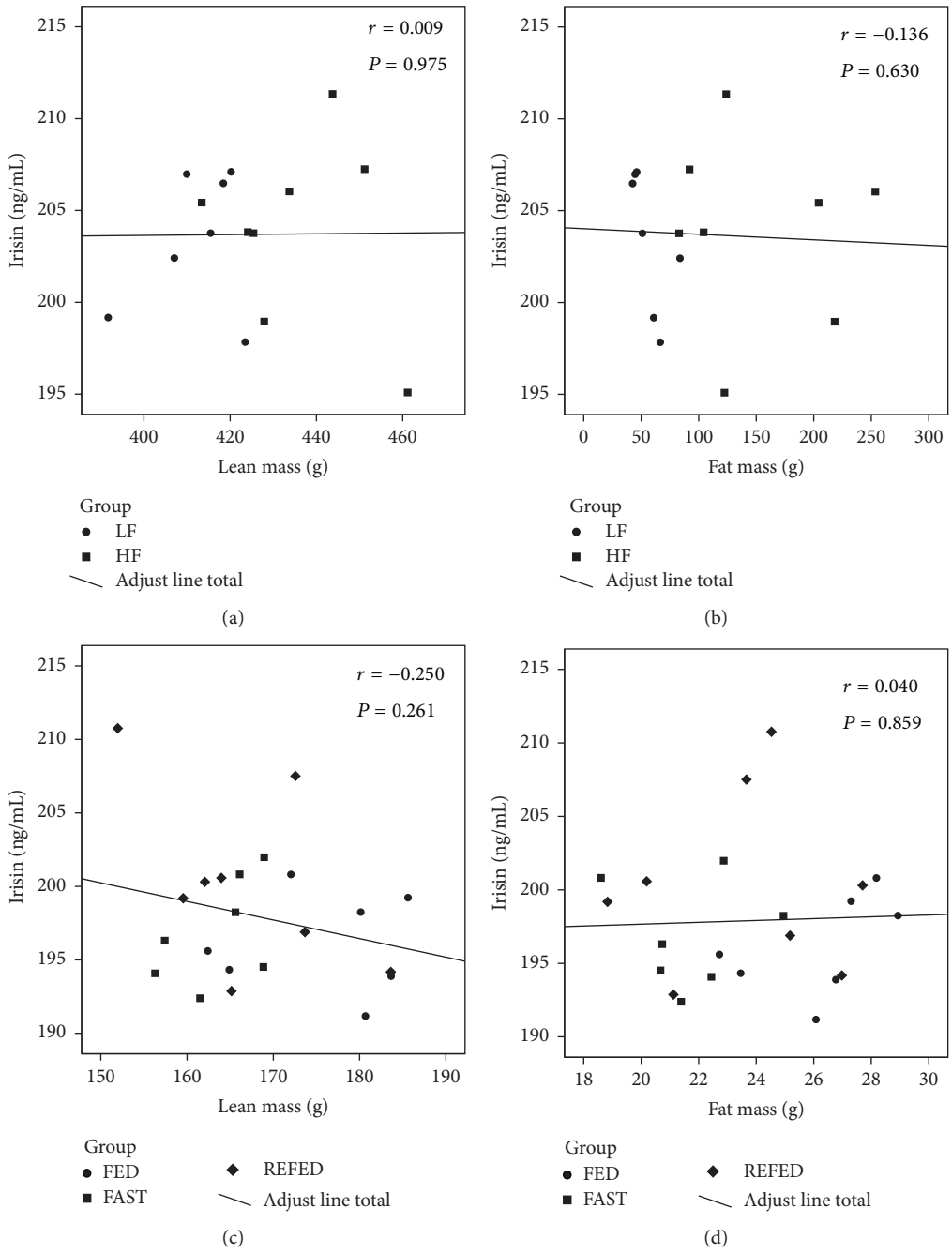

Figure 4: Correlation of irisin levels with body composition parameters. Correlation of irisin with lean mass (a) and fat mass (b) in DIO animals and with lean mass (c) and fat mass (d) in short-term changes of nutritional status.

mRNA expression of PGC1-α [41, 42], the proposed upstream regulator of irisin [8]. Moreover recently it was shown that leptin treatment slightly upregulates circulating irisin levels in ob/ob mice [47]. Therefore, we decided to investigate in depth the possible specific interaction between leptin and irisin. In order to test this interaction, we measured irisin levels in a model of hypoleptinemia performing a leptin replacement in ob/ob mice. Our data indicate that circulating levels of irisin remained unaltered after leptin administration in those mice in any experimental group. Therefore, these results suggest that serum irisin levels are not modulated by leptin on our experimental paradigms. Finally, we perform correlation studies of irisin with body composition, biochemical and hormonal parameters. In concordance with our previous results we do not find any correlation between them in most of the parameters studied (Figures 4(a), 4(b), 4(c), and 4(d); Figures 5(a), 5(c), 5(d), 5(e), 5(f), 5(h), and 5(i); Table 2); however, in spite of these negative results we detect a negative correlation between irisin and insulin in DIO animals as previously described in obese subjects [21]

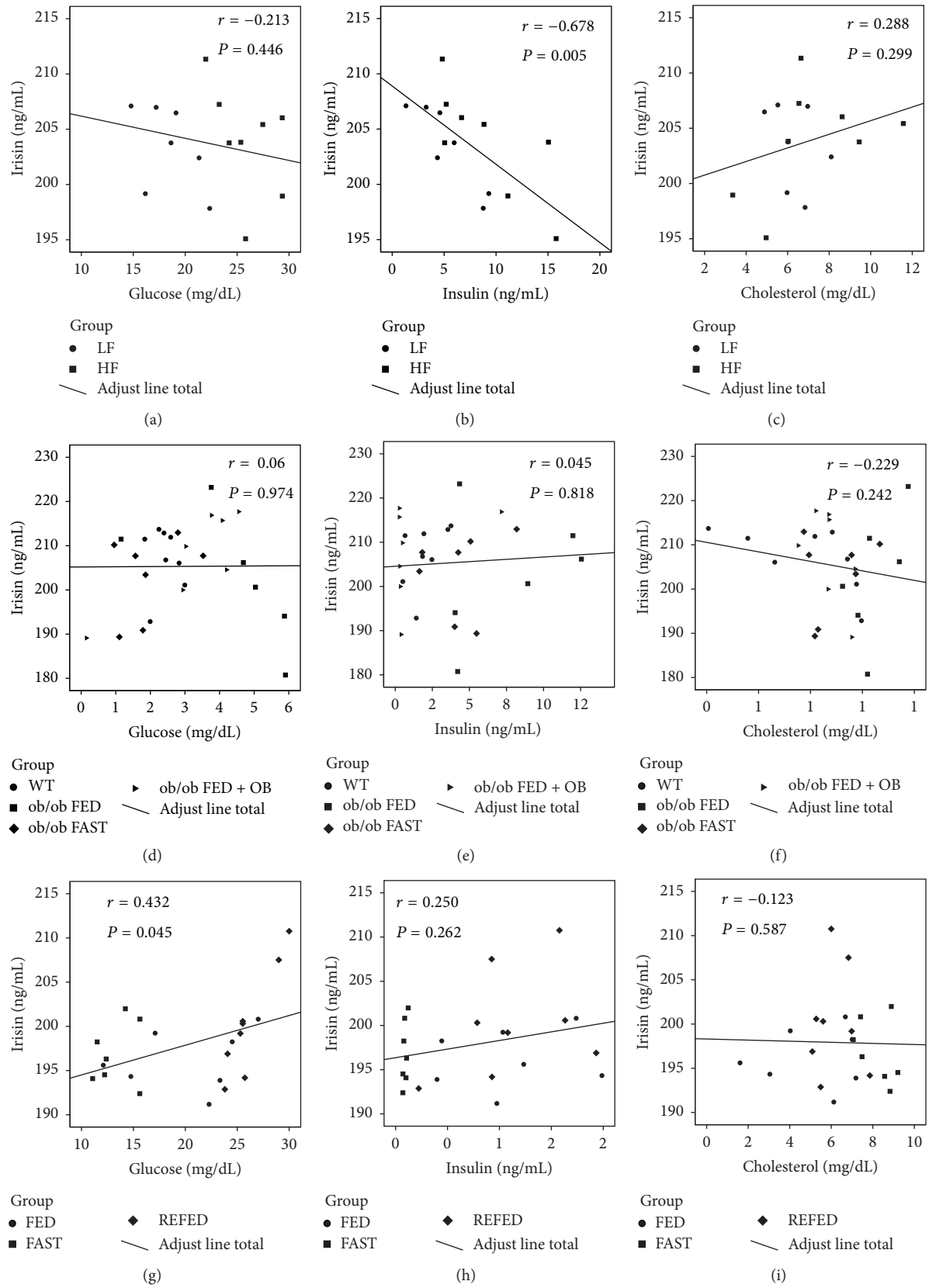

Figure 5: Correlation of irisin levels with biochemical and hormonal parameters. Correlation of irisin with glucose (a), insulin (b), and cholesterol (c) in DIO animals; with glucose (d), insulin (e), and cholesterol (f) in ob/ob mice; with glucose (g), insulin (h), and cholesterol (i) under short-term changes of nutritional status.

and a positive correlation between irisin and glucose under short-term changes in nutritional status (Figures 5(b) and 5(g); Table 2). These data indicate that irisin could play a role in states that involve impairments of glucose homeostasis.

In summary, we conclude that serum irisin levels seems in light of our present results not affected by obesity, nutritional status, or leptin in rodents. However, it is important to note that a limitation of the interpretation of the present results is that the data are based on a direct determination of irisin on serum samples without mechanism exploration or signal transduction studies of irisin with metabolism in obesity models. Further research on irisin will be necessary to clarify its precise role in the regulation of energy balance and its potential therapeutic use in obesity and its comorbidities.

Conflict of Interests

The authors declare that they have no conflict of interests in the authorship or publication of this paper.

Authors' Contribution

Cintia Folgueira and Estrella Sánchez-Rebordelo contributed equally to this work.

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Research Article

Inflammation, Oxidative Stress, and Antioxidants Contribute to Selected Sleep Quality and Cardiometabolic Health Relationships: A Cross-Sectional Study

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Sleep is vital for cardiometabolic health, but a societal shift toward poor sleep is a prominent feature of many modern cultures. Concurrently, factors such as diet and lifestyle have also changed and may mediate the relationship between sleep quality and cardiometabolic health. Objectives were to explore (1) the interrelationship and (2) mediating effect of inflammation, oxidative stress, and antioxidants on sleep quality and cardiometabolic health. Cross-sectional data from the US National Health and Nutritional Examination Survey 2005-06 (≥20 y; $N = 2{,}072$) was used. Cardiometabolic health was defined as per the Joint Interim Statement; overall sleep quality was determined from six sleep habits and categorized as good, fair, poor, and very poor. Fair quality sleepers had optimal inflammation, oxidative stress, and antioxidant levels. Inflammation was above the current clinical reference range across all sleep quality categories, while oxidative stress was only within the clinical reference range for fair sleep quality. Selected sleep quality-cardiometabolic health relationships were mediated by inflammation, oxidative stress, and antioxidants and were moderated by sex. Our results provide initial evidence of a potential role for inflammation, oxidative stress, and antioxidants in the pathway between poor sleep quality-cardiometabolic decline. Further prospective research is needed to confirm our results.

1. Introduction

Sleep is a vital human process needed for optimal immune, cardiometabolic, and cognitive health [1]. However, a societal trend toward less sleep and poorer quality sleep is a common feature in many developed countries [2, 3]. Whether sleep duration or quality is more important for health remains unclear, but some research suggests they modify each other's associations with health [4]. After adjusting for sleep duration, significant associations between sleep quality and cardiometabolic health were found in several population studies [4–6].

However, few studies have explored the association of sleep quality with inflammation, oxidative stress, and antioxidants levels [7, 8]. Observational studies support a modest-to-strong correlation between sleep quality measures and inflammation (i.e., C-Reactive Protein (CRP)) [9, 10], but the relationship may be sex-specific. For instance, Liu et al. [10] found that poor quality sleep was significantly associated with CRP in women, but not men. Additionally, the role of oxidative stress in sleep quality as well as the beneficial effect of antioxidant therapy has been demonstrated in obstructive sleep apnea patients [11]. Furthermore, the relationship between these factors (i.e., inflammation, oxidative stress, and antioxidants) and cardiometabolic health is also known and collectively suggests that abnormal levels of inflammation, oxidative stress, and insufficient antioxidants are associated with many age-related diseases, including diabetes, cardiovascular disease, and cancer [12]. Previously, we explored the interrelationship and mediating effect of inflammation (i.e., CRP), oxidative stress (i.e., γ-glutamyl transferase (GGT)), and antioxidants (i.e., bilirubin, carotenoids, uric acid, and vitamins A, C, D, and E) on *sleep duration* and cardiometabolic health [13]. However, the role of *sleep quality* within this context has not yet been explored. Thus, the purpose of this study is to (i) explore the interrelationship

between sleep quality and inflammation (CRP) [14], oxidative stress (GGT) [15], and antioxidants (bilirubin, carotenoids, uric acid, and vitamins A, C, D, and E) [16, 17] and (ii) quantify the indirect mediating effect of these factors on the sleep quality-cardiometabolic health relationships in free-living adults.

2. Methods

2.1. Participants. Data for this analysis was drawn from the US National Health and Nutrition Examination Survey (NHANES) [20]. NHANES is a series of nationally representative cross-sectional studies designed to assess the health and nutritional status of the US noninstitutionalized civilian population of all ages and ethnicities. Approximately 10,000 persons are sampled biannually, and data are collected from personal interviews, standardized physical examinations, and laboratory samples. NHANES 2005-2006 cycle, which had an initial sample of 10,348 individuals, was used in this study. Subsequent exclusions were made for age (<20 y: N = 5,369), pregnancy (N = 336), missing MetS components ($N_{\text{waist circumference}}$ = 455, $N_{\text{triglyceride}}$ = 196, $N_{\text{blood pressure}}$ = 79, $N_{\text{fasting plasma glucose}}$ = 1,826, and $N_{\text{HDL cholesterol}}$ = 0), and missing sleep quality variables (N = 15) for a final analytic sample of 2,072.

2.2. Metabolic Syndrome and Cardiometabolic Health. The Joint Interim Statement was used to define metabolic syndrome (MetS) [≥3 of elevated waist circumference: men (≥102 cm) and women (≥88 cm); elevated triglycerides or medication: ≥1.69 mM; low HDL cholesterol or medication: men (<1.04 mM) and women (<1.29 mM); elevated blood pressure or medication: systolic (≥130 mmHg) and/or diastolic (≥85 mmHg); and elevated fasting plasma glucose or medication use (≥5.6 mM)] [21]. These criteria were summed to determine the number of MetS components [0, 1, 2, 3, 4, 5]. Cardiometabolic health markers assessed were the individual MetS components included in the Joint Interim Statement.

2.3. Sleep Quality. The Sleep Disorders Questionnaire was administered to participants aged ≥16 y (limited to adults ≥20 y for our analysis), who reported their typical sleep habits for the past month [20]. This questionnaire contains items from two previously validated sleep questionnaires [22]. Overall sleep quality was determined from six questions: "How often did you have trouble falling asleep?"; "How often did you wake up during night and had trouble getting back to sleep?"; "How often did you wake up too early in morning and were unable to get back to sleep?"; "How often did you feel unrested during the day, no matter how many hours of sleep you had?"; "How often did you feel excessively or overly sleepy during day?"; and, "How often did you not get enough sleep?" Participants' responses to each question [0 = never; 1 = rarely (1 time a month); 2 = sometimes (2–4 times a month); 3 = often (5–15 times a month); and 4 = almost always (16–30 times a month)] were summed to obtain an index of overall sleep quality. The sleep quality score was subsequently categorized as good (0 to <3); fair (3 to <7); poor (7 to <12); and very poor (≥12 to 24) [3].

2.4. Mediators and Population Descriptors. Mediators considered in this study were CRP, GGT, bilirubin, carotenoids, uric acid, and vitamins A, C, D, and E and were obtained from laboratory files [23]. Blood samples were collected by certified phlebotomists, and we used the morning session's data which contained blood samples after an overnight fast [23]. Demographic variables used to describe the population include age (categorized as 20 to <40 y, 40 to <65 y, and ≥65 y), sex (men, women), ethnicity (Non-Hispanic White, Non-Hispanic Black, Mexican American, and Others), income (<$20,000, $20,000–44,999, and ≥$45,000), education (<high school, high school, and college), alcohol intake (0, <3, and ≥3 drinks per day), smoking history [current (if smoking now), past (if smoked ≥100 cigarettes in one's life but not current smoker), or never (if smoked <100 cigarettes in one's life)], and recreational physical activity (PA) adherence (none reported, <500 metabolic equivalent (MET)·min/wk and ≥500 MET·min/wk) [24, 25]. To estimate MET·min/wk for recreational PA adherence, we used NHANES suggested MET values, which were then categorized according to the PA guidelines for Americans [26].

2.5. Statistics. Mean and 95% confidence interval (CI) for continuous variables and frequency (%) and 95% CI for categorical variables were determined for each descriptor, by category of sleep quality. ANOVA (with *post hoc* Tukey's test) and χ^2 tests were used, as appropriate, to test for any differences in demographic and behavioral characteristics of sleep quality groups. The interrelationship between sleep quality and CRP, GGT, bilirubin, carotenoids, uric acid, and vitamins A, C, D, and E was determined and presented visually relative to the American Medical Association's clinical reference ranges [19]. Subsequent to this, the indirect mediation effect of CRP, GGT, bilirubin, carotenoids, uric acid, and vitamins A, C, D, and E on the sleep-cardiometabolic health relationship was estimated using (i) logistic regression for binary outcomes (i.e., MetS) and (ii) general linear models for the number of MetS components and individual cardiometabolic parameters [18].

Through a series of regression analyses, indirect mediation helps explain the underlying relationship between an exposure and an outcome variable through a third (mediatory) variable [18] (Figure 1). These relationships are depicted by four pathways: regression between exposure and mediator (path a); regression between mediator and outcome while adjusting for the exposure (path b); regression between exposure and outcome (path c); and regression between exposure and outcome while adjusting for the mediator (path c') [18]. Participants with a missing mediator variable were excluded from each regression analysis to ensure the products of ab and c-c' were equivalent [18]. The product of ab was subsequently used to classify indirect effects as large (≥0.25), moderate (≥0.09), modest (≥0.01), and weak (<0.01) [18]. To detect a moderate indirect effect with 80% power, n = 105 participants were needed in each sleep quality category. Significance for indirect effect was tested with the Sobel and Joint Significance tests.

To ensure the representativeness of the data, the medical exam sample weight from the demographics data file was

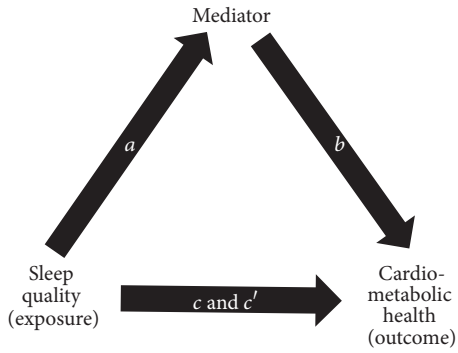


Figure 1: Multiple regression method of the indirect mediation model [18]. Path *a* indicates the path from sleep quality (exposure) to mediator (i.e., inflammation, oxidative stress, and antioxidant). Path *b* indicates the path from mediator to outcome (i.e., metabolic syndrome (MetS), number of MetS components, and individual MetS components) controlling for the mediator. Path *c* indicates the path from exposure to outcome. Path c' indicates the path from exposure to outcome controlling for the mediator.

used to weight all analyses [20]. All analyses were conducted in SAS v9.3 (Cary, NC, U.S.A) with statistical significance set at an α of 0.05.

3. Results

Table 1 describes the characteristics of the US adult population, stratified by sleep quality categories. In general, sleep quality varied by sex, age, ethnicity, education, recreational PA, and smoking. Specifically, middle aged adults (40 to <65 y) tended to have very poor quality sleep, while older adults reported good quality sleep compared to other sleep quality categories. Very poor sleep quality was also more common among women, Non-Hispanic Whites, and current smokers. Compared to good and very poor quality sleepers, both fair and poor sleepers attained more recreational PA, half of whom, approximately, met the PA guidelines for Americans.

Figure 2 illustrates the interrelationship between sleep quality and inflammation (a), oxidative stress (b), and antioxidants (c)–(i) with clinical reference ranges shaded in gray. Those reporting fair, but not good, quality of sleep had the optimal inflammation and oxidative stress profiles. However, inflammation was above the clinical reference range across all sleep quality categories. For GGT, all levels of sleep quality except those with very poor quality sleep had mean values within the clinical reference range. All antioxidants were within the clinical reference range and were generally optimal among fair or poor quality sleepers. CRP and vitamin C levels were statistically different for fair versus very poor quality sleep.

Overall, vitamins A and C were modest mediators of the sleep quality-MetS and sleep quality-number of MetS components relationships (Table 2). Further exploration of the indirect effect revealed these antioxidants were also moderate (≥0.09) mediators of the sleep quality-waist circumference and sleep quality-systolic blood pressure relationships (Table 3). Additionally, the sleep quality-waist circumference and sleep quality-diastolic blood pressure relationships were moderately mediated by CRP.

While some minor differences between men and women were observed for the sleep quality-MetS and sleep quality-number of MetS components relationships (men: Table 4; women: Table 5), the differences were larger for some individual components (men: Table 6; women: Table 7). In women, CRP, uric acid, and vitamin C were large mediators of the sleep quality-waist circumference relationship and moderately mediated by carotenoids and GGT. Uric acid was also a large mediator of the sleep quality-systolic blood pressure relationship in women, while CRP and carotenoids were moderate mediators. Finally, vitamin C was a moderate mediator of the sleep quality-diastolic blood pressure relationship in women.

4. Discussion

We found that very poor sleep quality was more common amongst women, Non-Hispanic Whites, current smokers, and middle aged adults (40 to <65 y), whereas older adults were more likely to report good quality sleep. Optimal inflammation and oxidative stress profiles were found amongst fair quality sleepers, while some antioxidants were also optimal amongst fair and poor quality sleepers. While most sleep quality-MetS or sleep quality-number of MetS components relationship were not significant, selected sleep quality-cardiometabolic health relationships were moderately mediated by CPR and vitamins A and C. Additionally, in women only, the indirect effects were moderate-to-large for CRP, GGT, carotenoids, uric acid, and vitamin C.

4.1. Inflammation, Oxidative Stress, and Antioxidants Profiles. Our finding of optimal inflammation, oxidative stress, and antioxidant levels amongst the fair or poor sleep quality categories was surprising, as we expected optimal levels amongst those with at least good sleep quality. To date, relatively few studies have investigated the interrelationship between inflammation, oxidative stress, and antioxidants with sleep quality [7, 8, 27–29]. In one such study (n = 24), adherence to a kiwi diet (2 kiwi/night for 4 weeks), a fruit rich in vitamins C and E and serotonin, improved sleep onset and duration [27]. Another similar study (n = 20) found that tart cherry juice, rich in vitamins A and C, improved sleep quality, suggesting increased melatonin levels as a possible mechanism for this effect [28]. However, neither of these studies measured serum antioxidant levels [27, 28]. In sleep disordered populations, elevated levels of inflammation and oxidative stress are common features [29]; and overall antioxidant capacity tends to decrease with age [30].

Another possible explanation is the definition of sleep quality, which is based on the six questions that were assigned equal weights [3]. In a previous analysis, we found that the odds of MetS varied between questions, with no significant relationship for getting enough sleep (OR: 1.06) and roughly twofold increases for trouble falling asleep, feeling overly sleepy, and waking up during the night, after adjusting for

Table 1: Characteristics of the US adult population ≥20 years of age.

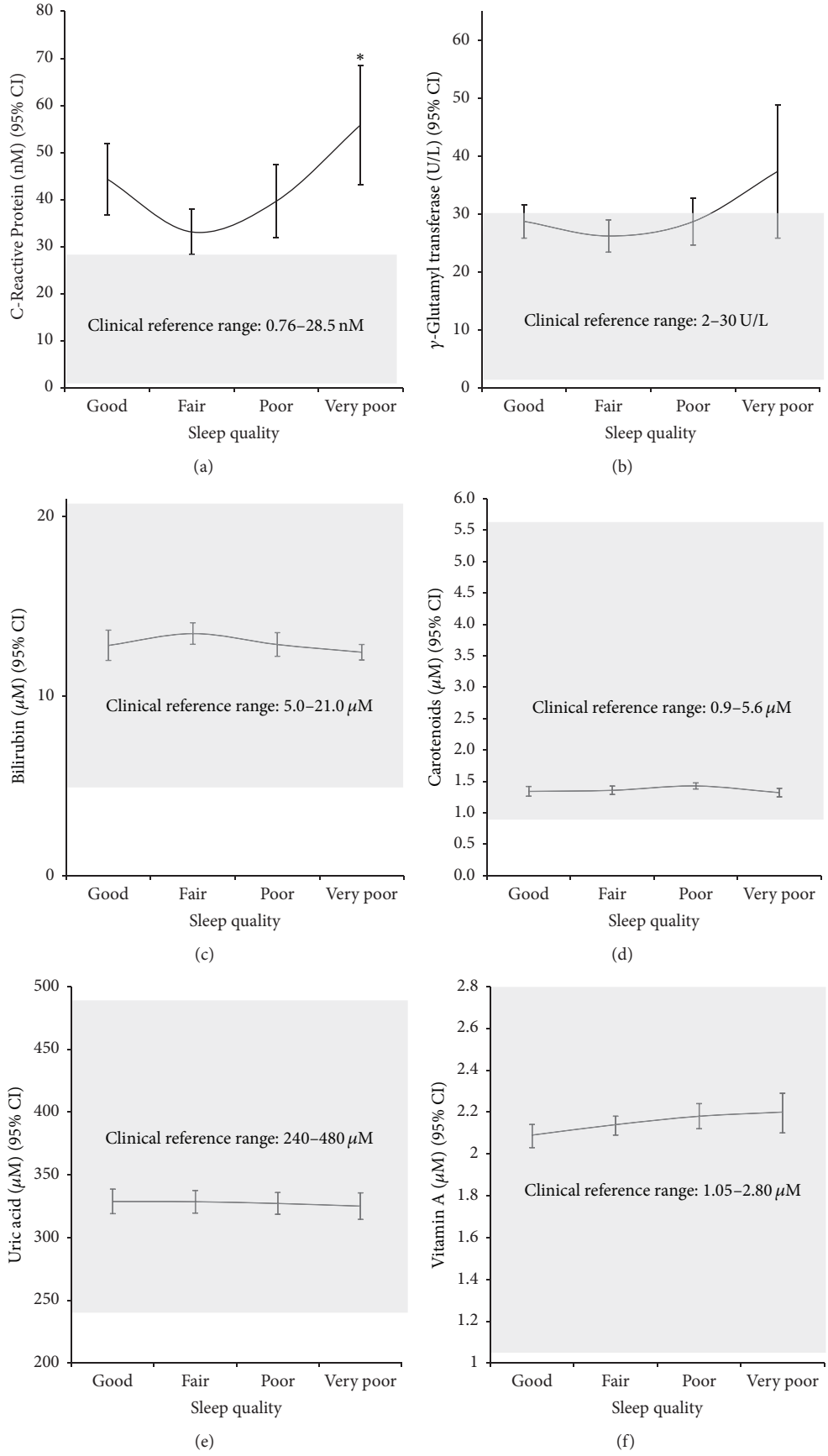
Characteristics	Sleep quality				p value
	Good (n = 428)	Fair (n = 488)	Poor (n = 579)	Very poor (n = 577)	
Age (mean (95% CI))	51.5 (48.7, 54.3)	48.9 (46.2, 51.6)	46.6 (44.4, 48.8)	46.7 (44.5, 48.9)	<0.05
Age categories (% (95% CI))					
20 to <40 years	28.2 (21.7, 34.7)	33.9 (28.6, 39.2)	36.8 (31.8, 41.8)	33.7 (29.1, 38.4)	<0.05
40 to <65 years	45.9 (40.4, 51.5)	44.8 (39.2, 50.4)	47.1 (44.1, 50.1)	51.3 (45.7, 56.9)	
≥65 years	25.9 (20.3, 31.4)	21.3 (15.9, 26.6)	16.1 (12.2, 20.0)	15.0 (10.0, 19.9)	
Sex					
Men	60.8 (55, 66.6)	51.5 (46.5, 56.4)	51.1 (47.5, 54.8)	43.4 (39.5, 47.4)	<0.05
Women	39.2 (33.4, 45.0)	48.5 (43.6, 53.5)	48.9 (45.2, 52.5)	56.6 (52.6, 60.5)	
Ethnicity					
Non-Hispanic White	58.1 (49.1, 67.2)	73.8 (66.7, 80.9)	75.2 (67.4, 82.9)	73.6 (68.3, 78.9)	<0.05
Non-Hispanic Black	15.6 (10.7, 20.5)	10.0 (5.5, 14.6)	8.6 (5.1, 12.2)	13.0 (8.9, 17.1)	
Mexican American	14.8 (9.1, 20.6)	7.0 (3.5, 10.6)	6.6 (4.6, 8.6)	5.3 (3.2, 7.3)	
Others	11.4 (6.0, 16.8)	9.2 (3.6, 14.7)	9.6 (5.1, 14.1)	8.1 (5.0, 11.3)	
Education					
<High school	26.1 (18.7, 33.5)	16.5 (11.2, 21.8)	14.2 (10.9, 17.5)	16.8 (12.5, 21.1)	<0.05
High school	25.5 (19.0, 32.1)	24.9 (20.1, 29.7)	24.4 (20.2, 28.6)	27.7 (24.0, 31.4)	
College	48.4 (36.2, 60.6)	58.6 (51.5, 65.7)	61.4 (56.0, 66.8)	55.5 (50.0, 61.0)	
Income					
<$20,000	16.8 (12.0, 21.6)	14.7 (10.8, 18.6)	12.9 (9.2, 16.7)	18.8 (14.1, 23.6)	NS
$20,000–44,999	33.7 (25.8, 41.5)	33.1 (27.2, 39.0)	30.3 (23.6, 37.0)	31.5 (24.0, 39.0)	
≥$45,000	49.5 (39.5, 59.6)	52.2 (45.6, 58.9)	56.8 (49, 64.5)	49.7 (42.1, 57.3)	
Smoking					
None	52.1 (43.0, 61.3)	47.7 (40.8, 54.5)	52.9 (47.9, 58)	42.8 (36.6, 49.0)	<0.05
Current	20.7 (14.7, 26.8)	20.6 (14.9, 26.4)	22.1 (17.1, 27.2)	34.3 (29.7, 39.0)	
Past	27.1 (21.5, 32.8)	31.7 (26.8, 36.6)	24.9 (20.8, 29.0)	22.8 (19.3, 26.4)	
Alcohol intake					
0 drinks/d	36.6 (30.9, 42.3)	29.8 (23.3, 36.3)	28.1 (22.2, 34.0)	33.6 (29.4, 37.8)	NS
<3 drinks/d	41.3 (34.4, 48.3)	46.3 (39.5, 53.1)	44.8 (39.0, 50.5)	39.7 (34.5, 44.8)	
≥3 drinks/d	22.1 (16.9, 27.3)	23.9 (19.2, 28.6)	27.1 (22.4, 31.9)	26.8 (21.8, 31.7)	
Recreational physical activity					
None reported	41.2 (34.2, 48.1)	28.2 (24.2, 32.3)	28.2 (22.9, 33.5)	35.6 (31.2, 39.9)	<0.05
<500 MET·min/w	16.4 (11.3, 21.5)	22.2 (17.8, 26.6)	25.1 (20.2, 30.1)	22.4 (18.3, 26.5)	
≥500 MET·min/w	42.4 (36.9, 48.0)	49.6 (44.1, 55.1)	46.6 (41.9, 51.4)	42.1 (35.2, 49.0)	

Mean (95% CI) for continuous variables and % (95% CI) for categorical variables. Overall sleep quality was calculated based on six questions on sleep habits of participants and categorized into quartiles: good (<3), fair (≥3 to 7), poor (≥7 to 12), and very poor (≥12). $p < 0.05$, two-sided; ANOVA or Chi-square, as appropriate. NS: not significant. Sum of weights = 95,276,598.

age, sex, ethnicity, education, income, smoking, recreational physical activity, and sleep duration (unpublished data). Therefore, future work may benefit from the development of a recalibrated (weighted index) of sleep quality.

4.2. Indirect Mediation Effect. Evaluating the indirect effect of inflammation, oxidative stress, and antioxidants on the relationship between sleep quality and cardiometabolic health was the second aim of our study. Although our overall findings were weak or modest for the sleep quality-MetS and sleep quality-number of MetS components relationships, moderate indirect effects by CPR and vitamin C on the sleep quality-waist circumference and vitamin A for sleep quality-systolic blood pressure relationships warrant discussion.

4.2.1. Waist Circumference. Although the relationship between poor sleep quality and body weight is well known [31], little work has been done to examine the association with sleep quality. Positive associations between waist circumference and CRP or vitamin C have nonetheless been found [32, 33]. Evidence also suggests that sleep architecture may be altered in individuals with obesity; that is, rapid eye movement stage of sleep may occur earlier compared to normal weight individuals [34]. In sleep disordered populations, the prevalence of obesity is high; and both sleep quality and obesity have been independently linked to increased inflammation and decreased antioxidants [7, 14, 31]. For instance, a large Finnish study reported that CRP was elevated (≥9.52 nM) among men reporting frequent sleep



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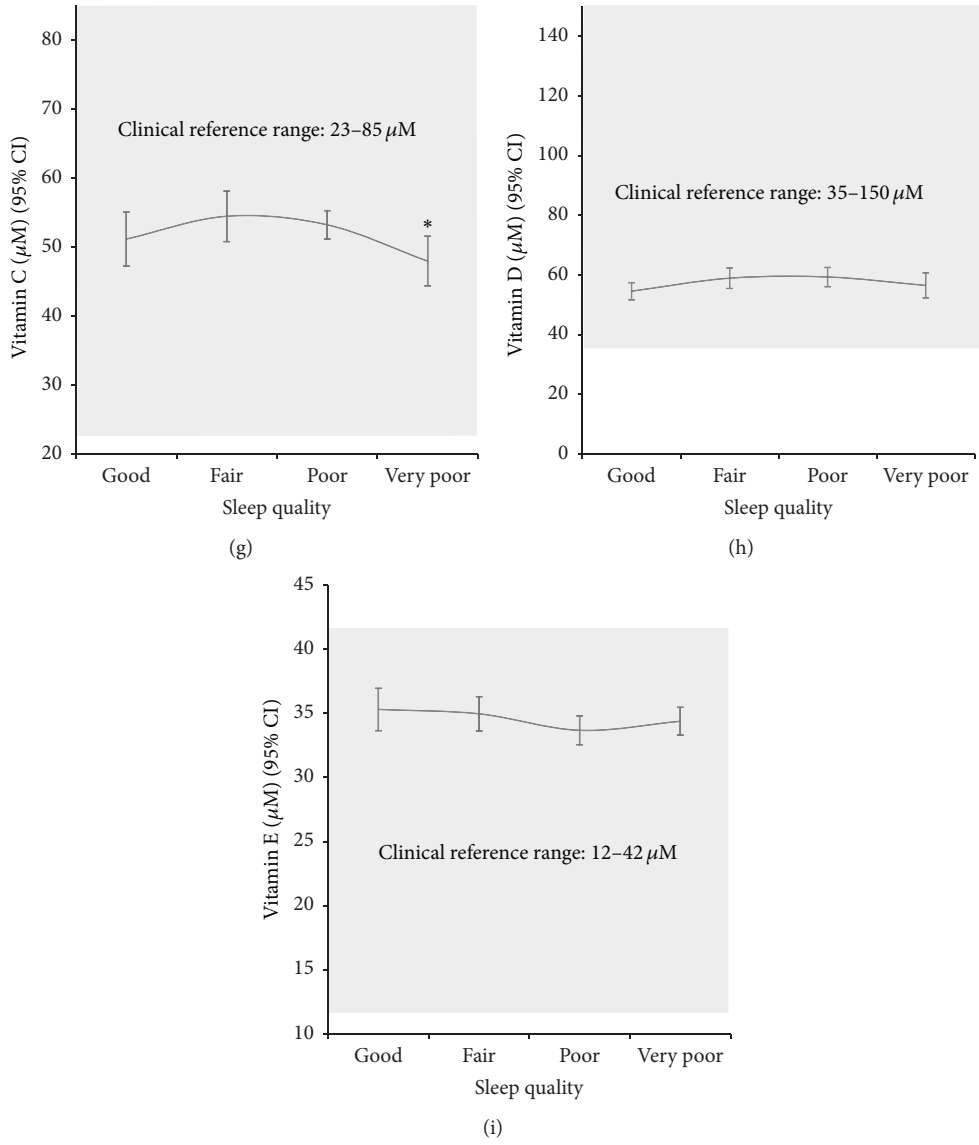

Figure 2: Interrelationship between sleep quality and inflammation (a), oxidative stress (b), and antioxidants ((c)–(i)). Gray shaded areas are clinical reference ranges [19]. $^{*}p < 0.05$ versus fair sleep quality.

Table 2: Indirect effect of mediators on the sleep quality-metabolic syndrome and sleep quality-number of MetS components relationship.

Mediator	c-c'	ab (95% CI_{sobel})	
	MetS	MetS	Number of MetS components
C-Reactive Protein (nM)	0.012	0.014 (−0.007, 0.035)	0.011 (−0.004, 0.026)
γ-Glutamyl transferase (U/L)	0.022	0.042 (−0.006, 0.090)	0.011 (−0.003, 0.025)
Bilirubin (μM)	0.006	0.006 (−0.001, 0.013)	0.006 (−0.001, 0.012)
Carotenoids (μM)	0.002	0.002 (−0.012, 0.016)	0.001 (−0.010, 0.013)
Uric acid (μM)	−0.009	−0.011 (−0.038, 0.017)	−0.008 (−0.030, 0.013)
Vitamin A (μM)	0.017	**0.016** (**0.001**, **0.031**)*	**0.015** (**0.001**, **0.028**)*
Vitamin C (μM)	0.019	**0.018** (**0.003**, **0.034**)*	**0.014** (**0.002**, 0.026)*
Vitamin D (nM)	−0.004	−0.005 (−0.024, 0.014)	−0.004 (−0.021, 0.013)
Vitamin E (μM)	−0.015	−0.016 (−0.040, 0.008)	−0.014 (−0.035, 0.006)

MetS is metabolic syndrome. c-c' and ab are indirect effect. *Significant for Joint test, and bold means significant for Sobel test.

Table 3: Indirect effect of mediators on the sleep quality-individual MetS component relationship.

Mediator	*ab* (95% CI_{sobel})					
	Waist circumference	Systolic blood pressure	Diastolic blood pressure	Triglycerides	HDL cholesterol	Fasting plasma glucose
C-Reactive Protein (nM)	0.204 (−0.004, 0.412)*	0.058 (−0.023, 0.140)	0.035 (−0.009, 0.078)*	0.005 (−0.002, 0.011)	−0.001 (−0.003, 0.000)	0.009 (−0.002, 0.020)*
γ-Glutamyl transferase (U/L)	0.101 (−0.025, 0.226)	0.101 (−0.037, 0.240)	0.049 (−0.013, 0.112)	0.010 (−0.004, 0.023)	−0.001 (−0.002, 0.001)	0.013 (−0.005, 0.032)
Bilirubin (μM)	0.016 (−0.110, 0.142)	0.014 (−0.06, 0.088)	−0.002 (−0.012, 0.008)	0.000 (0.000, 0.000)	0.000 (−0.003, 0.002)	0.000 (−0.008, 0.007)
Carotenoids (μM)	0.094 (−0.023, 0.211)	0.071 (−0.01, 0.151)	−0.010 (−0.034, 0.014)	0.001 (−0.001, 0.004)	−0.001 (−0.003, 0.000)	0.004 (−0.004, 0.012)
Uric acid (μM)	−0.115 (−0.411, 0.181)	−0.051 (−0.192, 0.09)	−0.013 (−0.052, 0.027)	−0.004 (−0.016, 0.007)	0.002 (−0.003, 0.006)	0.000 (−0.003, 0.002)
Vitamin A (μM)	0.046 (−0.022, 0.115)	**0.158 (0.023, 0.294)***	0.001 (−0.034, 0.037)	**0.019 (0.002, 0.036)***	0.001 (−0.001, 0.002)	0.000 (−0.005, 0.006)
Vitamin C (μM)	**0.227 (0.039, 0.414)***	0.049 (−0.028, 0.125)	**0.076 (0.008, 0.145)***	**0.010 (0.001, 0.020)***	**−0.005 (−0.009, −0.001)***	**0.011 (0.003, 0.018)***
Vitamin D (nM)	−0.057 (−0.292, 0.178)	−0.034 (−0.143, 0.074)	−0.008 (−0.040, 0.023)	−0.002 (−0.008, 0.005)	0.001 (−0.003, 0.004)	−0.004 (−0.018, 0.011)
Vitamin E (μM)	−0.064 (−0.160, 0.032)	−0.099 (−0.266, 0.068)	−0.035 (−0.092, 0.022)	−0.027 (−0.067, 0.012)	0.000 (−0.001, 0.001)	−0.010 (−0.025, 0.005)

ab is indirect effect. *Significant for Joint test, and bold means significant for Sobel test.

Table 4: Indirect effect of mediators on the sleep quality-metabolic syndrome and sleep quality-number of MetS components relationship for men.

Mediator	c-c'	*ab* (95% CI_{sobel})	
	MetS	MetS	Number of MetS components
C-Reactive Protein (nM)	0.002	0.001 (−0.006, 0.008)	0.002 (−0.006, 0.010)
γ-Glutamyl transferase (U/L)	0.024	0.042 (−0.013, 0.097)	0.014 (−0.003, 0.031)
Bilirubin (μM)	0.001	0.000 (−0.013, 0.013)	0.000 (−0.011, 0.011)
Carotenoids (μM)	0.010	−0.014 (−0.032, 0.004)	−0.011 (−0.024, 0.002)
Uric acid (μM)	0.013	−0.001 (−0.06, 0.058)	−0.001 (−0.041, 0.039)
Vitamin A (μM)	0.017	0.015 (−0.004, 0.034)	**0.014 (0.000, 0.028)***
Vitamin C (μM)	0.012	0.007 (−0.021, 0.036)	0.005 (−0.016, 0.026)
Vitamin D (nM)	−0.009	−0.010 (−0.024, 0.004)	−0.011 (−0.024, 0.002)
Vitamin E (μM)	−0.018	**−0.029 (−0.057, 0.000)***	**−0.026 (−0.050, −0.002)***

MetS is metabolic syndrome. *ab* is indirect effect. *Significant for Joint test, and bold means significant for Sobel test.

Table 5: Indirect effect of mediators on the sleep quality-metabolic syndrome and sleep quality-number of MetS components relationship for women.

Mediators	c-c'	*ab* (95% CI_{sobel})	
	MetS	MetS	Number of MetS components
C-Reactive Protein (nM)	0.036	**0.044 (0.011, 0.078)***	**0.035 (0.012, 0.059)***
γ-Glutamyl transferase (U/L)	0.042	0.049 (−0.012, 0.109)*	0.017 (−0.001, 0.035)*
Bilirubin (μM)	0.003	0.003 (−0.005, 0.011)	0.005 (−0.005, 0.015)
Carotenoids (μM)	0.012	0.020 (0.000, 0.040)	0.017 (0.000, 0.033)
Uric acid (μM)	0.035	**0.056 (0.007, 0.105)***	**0.043 (0.005, 0.08)***
Vitamin A (μM)	0.022	0.025 (−0.006, 0.055)	0.021 (−0.005, 0.047)
Vitamin C (μM)	0.031	**0.032 (0.008, 0.055)***	**0.022 (0.004, 0.041)***
Vitamin D (nM)	0.006	0.013 (−0.036, 0.062)	0.010 (−0.028, 0.048)
Vitamin E (μM)	−0.014	**−0.032 (−0.062, −0.002)***	−0.003 (−0.030, 0.024)

MetS is metabolic syndrome. *ab* is indirect effect. *Significant for Joint test, and bold means significant for Sobel test.

Table 6: Indirect effect of mediators on the sleep quality-individual MetS component relationship for men.

Mediators	*ab* (95% CI_{sobel})					
	Waist circumference	Systolic blood pressure	Diastolic blood pressure	Triglycerides	HDL cholesterol	Fasting plasma glucose
C-Reactive Protein (nM)	0.046 (−0.159, 0.251)	0.01 (−0.042, 0.062)	0.009 (−0.033, 0.051)	0.000 (−0.002, 0.003)	0.000 (−0.003, 0.002)	0.001 (−0.003, 0.006)
γ-Glutamyl transferase (U/L)	0.096 (−0.015, 0.206)	0.095 (−0.028, 0.218)	0.075 (−0.023, 0.173)	0.014 (−0.005, 0.033)	0.001 (−0.001, 0.002)	0.019 (−0.009, 0.047)
Bilirubin (μM)	0.001 (−0.099, 0.100)	0.001 (−0.074, 0.075)	0.000 (−0.031, 0.031)	0.000 (−0.004, 0.004)	0.000 (−0.002, 0.002)	0.000 (−0.003, 0.003)
Carotenoids (μM)	−0.12 (−0.264, 0.024)	−0.066 (−0.163, 0.03)	0.001 (−0.028, 0.03)	0.002 (−0.003, 0.008)	0.003 (−0.001, 0.006)	−0.006 (−0.014, 0.003)
Uric acid (μM)	−0.010 (−0.535, 0.515)	−0.003 (−0.12, 0.115)	−0.004 (−0.141, 0.133)	0.000 (−0.021, 0.020)	0.000 (−0.007, 0.008)*	−0.001 (−0.012, 0.009)
Vitamin A (μM)	−0.041 (−0.148, 0.067)	0.093 (−0.008, 0.194)*	0.031 (−0.032, 0.093)	**0.024** (**0.002**, **0.045**)*	0.002 (−0.001, 0.004)	0.000 (−0.008, 0.007)
Vitamin C (μM)	0.067 (−0.202, 0.336)	0.021 (−0.081, 0.123)	0.029 (−0.087, 0.144)	0.004 (−0.011, 0.019)	−0.001 (−0.007, 0.004)*	0.006 (−0.006, 0.018)
Vitamin D (nM)	−0.169 (−0.364, 0.025)	−0.048 (−0.12, 0.024)	−0.042 (−0.121, 0.037)	−0.009 (−0.020, 0.002)	0.004 (0.000, 0.008)*	−0.012 (−0.027, 0.004)
Vitamin E (μM)	−0.107 (−0.228, 0.013)*	−0.114 (−0.243, 0.016)*	−0.105 (−0.204, −0.007)*	**−0.059** (**−0.115**, **−0.004**)*	0.001 (−0.001, 0.004)	−0.010 (−0.024, 0.004)

ab is indirect effect. *Significant for Joint test, and bold means significant for Sobel test.

Table 7: Indirect effect of mediators on the sleep quality-individual MetS component relationship for women.

Mediators	*ab* (95% CI_{sobel})					
	Waist circumference	Systolic blood pressure	Diastolic blood pressure	Triglycerides	HDL cholesterol	Fasting plasma glucose
C-Reactive Protein (nM)	**0.539 (0.204, 0.875)***	**0.189 (0.023, 0.355)***	0.093 (−0.005, 0.190)	0.017 (0.000, 0.034)*	−0.004 (−0.009, 0.001)	**0.030 (0.011, 0.048)**
γ-Glutamyl transferase (U/L)	**0.143 (−0.031, 0.317)***	0.188 (−0.006, 0.382)	0.031 (−0.014, 0.076)	0.011 (−0.004, 0.026)	0.000 (−0.002, 0.002)	0.015 (−0.004, 0.034)
Bilirubin (μM)	0.051 (−0.059, 0.161)	0.002 (−0.06, 0.063)	0.018 (−0.025, 0.060)	0.004 (−0.004, 0.012)	−0.002 (−0.006, 0.002)*	−0.002 (−0.010, 0.007)
Carotenoids (μM)	**0.199 (0.001, 0.397)**	0.133 (−0.005, 0.272)*	−0.019 (−0.059, 0.022)	0.003 (−0.001, 0.008)	−0.004 (−0.009, 0.000)	0.012 (−0.002, 0.025)
Uric acid (μM)	**0.474 (0.058, 0.890)***	**0.359 (0.065, 0.654)***	−0.022 (−0.075, 0.032)	**0.016 (0.002, 0.031)***	−0.003 (−0.007, 0.000)*	**0.013 (−0.002, 0.028)***
Vitamin A (μM)	0.073 (−0.044, 0.191)	0.276 (−0.049, 0.602)	−0.052 (−0.145, 0.042)	0.022 (−0.005, 0.048)	0.004 (−0.001, 0.009)	0.000 (−0.008, 0.009)
Vitamin C (μM)	**0.445 (0.162, 0.728)***	0.024 (−0.199, 0.246)	**0.127 (0.020, 0.234)***	**0.014 (0.003, 0.025)***	**−0.007 (−0.013, −0.002)**	**0.017 (0.001, 0.034)***
Vitamin D (nM)	0.140 (−0.373, 0.654)	0.054 (−0.234, 0.342)	0.006 (−0.027, 0.039)	0.002 (−0.006, 0.011)	−0.002 (−0.007, 0.004)*	0.004 (−0.020, 0.028)
Vitamin E (μM)	−0.015 (−0.154, 0.123)	−0.006 (−0.327, 0.315)	−0.001 (−0.034, 0.033)	−0.005 (−0.046, 0.037)	0.000 (−0.001, 0.001)	−0.009 (−0.040, 0.022)

ab is indirect effect. *Significant for Joint test, and bold means significant for Sobel test.

disturbances, even after adjusting for BMI [35]. Vitamin C supplementation, on the other hand, improved endothelial function in patients with obstructive sleep apnea [36], a condition associated with increased bodyweight [15]. In rodents, the beneficial effects of vitamin C on weight gain and the absorption of lipids has been demonstrated [37, 38], and some more limited research suggests that vitamin C supplementation could help reduce adiposity through alterations in gene expression [39]. While the direct inflammatory and antioxidant mechanisms purported to influence the relationship between poor sleep quality and obesity remain unknown, our study provides initial evidence of a mediating role of CRP and vitamin C on the relationship between sleep quality and abdominal obesity.

4.2.2. Systolic Blood Pressure. While evidence suggests vitamin C could improve endothelial function [36], we did not find that it significantly mediated the sleep quality-systolic blood pressure relationship; rather, we found vitamin A moderately mediated the sleep quality-systolic blood pressure relationship. In mice, deficiency in vitamin A decreased nonrapid eye movement (REM) sleep stages 3 and 4 (i.e., deep or slow wave sleep); and evidence in humans suggest that vitamin A has an important role in sleep homeostasis [40].

4.3. Sex-Stratified Indirect Mediation Effect. We found that CRP, GGT, carotenoids, uric acid, and vitamin C were moderate-to-large mediators of selected sleep-cardiometabolic health relationships in women only. Others have found similar sex differences between inflammation, oxidative stress, antioxidants, and cardiometabolic dysfunction [17, 41]. Increased systematic inflammation is a particular concern in aging women since poor sleep duration, poor sleep quality, lack of social interactions, and abdominal adiposity have all been associated with inflammation [42, 43]. Additionally, Okun et al. [44] demonstrated a link between inflammation and poor sleep quality, proposing that chronic low-grade inflammation as a result of poor sleep quality in early adulthood (i.e., 20s) may predispose women to inflammation-related diseases in middle-adulthood. Indeed, the relationship between inflammation, oxidative stress, antioxidants, and age-related diseases warrants further study in women. Changes in female sex hormone levels, for instance, are associated with longer sleep duration and poorer sleep quality [45], while risk of cardiometabolic disease [46] and adiposity [47] also changes with circulating testosterone, estradiol, and sex hormone binding globulin levels. Taken together, our findings suggest that strategies to improve dietary or sleep habits may be valuable for the cardiometabolic health of women.

4.4. Strengths and Limitations. There are several study limitations that warrant mention. First, given the preliminary and cross-sectional nature of our findings, future longitudinal studies are needed to evaluate evidence for a cause-and-effect relationship. Second, in applying our study exclusion criteria, our final analytic sample was limited to less than 50% of the initial adult sample. Third, since all sleep quality variables were self-reported, they are susceptible to recall and response biases. However, we are not aware of a comprehensive population-based dataset that contains objective measures of sleep quality along with other physical measures necessary for this research question [i.e., accelerometry, polysomnography, or electroencephalogram,

serum biomarkers, and cardiometabolic health information]. We were also unable to study the effect of adipokines, such as leptin and adiponectin, which have been linked to poor sleep quality [48] and obesity [49]. Moreover, this work is limited to a select number of biomarkers for inflammation and oxidative stress. Future work would therefore benefit from the inclusion of interleukin-6, tumour necrosis factor-α, and malondialdehyde [50–52] in participants with diagnosed inflammatory diseases. Finally, since we used single measurements of exposure, outcome, and mediators, we are unable to account for potential changes in modifiable habits (i.e., sleep and diet) and their effect on our outcomes.

5. Conclusions

Improving sleep quality may minimize cardiometabolic decline though mechanisms involving inflammation, oxidative stress, and antioxidants. Further prospective work is needed to extend our understanding of the multiple pathways that may govern these factors.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Symmetric Dimethylarginine Is Not Associated with Cumulative Inflammatory Load or Classical Cardiovascular Risk Factors in Rheumatoid Arthritis: A 6-Year Follow-Up Study

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Symmetric dimethylarginine (SDMA) indirectly inhibits nitric oxide (NO) synthesis and predicts cardiovascular and all-cause mortality in high-risk patients. The aim of our study was to investigate the associations of cumulative inflammatory burden (assessed by serial measurements of inflammatory markers) and classical cardiovascular (CV) disease risk factors with SDMA in RA patients. 201 RA patients (155 females, median age 67 (59–73)) were assessed at baseline (2006). Classical CV disease risk factors were recorded and systemic inflammation was determined by the measurement of C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR). At follow-up (2012) SDMA levels were measured by enzyme-linked immunosorbent assay. Mean SDMA levels in RA population were 0.40 (0.40–0.53) μmol/L. No significant association between SDMA and cumulative inflammatory load was established in the analysis. SDMA levels were not found to be significantly related to CV disease risk factors. We explored the potential relationship between SDMA and cumulative inflammatory burden in patients with RA and obtained negative results. SDMA did not relate to CV disease risk factors in our population and its clinical significance as a surrogate marker of endothelial dysfunction in patients with RA remains to be determined.

1. Introduction

There is a substantial amount of evidence suggesting that cardiovascular (CV) disease is the most common cause of premature mortality in patients with rheumatoid arthritis (RA) [1]. Even after adjusting for traditional CV risk factors, the magnitude of CV risk in RA is increased by approximately 50% compared to the general population [2]. Components of cardiometabolic syndrome such as dyslipidemia, insulin resistance, and hypertension are more common in patients with RA leading to endothelial dysfunction and coronary artery disease [3].

It is well-recognised that the endothelium is the key regulator of vascular function by controlling vascular tone, smooth muscle proliferation, platelet aggregation, and migration of adhesion molecules [4]. Inflammation in RA has a systemic nature and inflammatory molecules and cytokines exhibit deleterious effects on the vasculature resulting in endothelial dysfunction. Derangement of endothelial homeostasis is an early preclinical marker of atherosclerosis and several studies have demonstrated that endothelial dysfunction is more prevalent in RA patients compared to controls [5]. The damaged endothelium disrupts the metabolism of nitric oxide (NO) which is the most important vasoactive

agent for the maintenance of basal vasodilator tone and vascular patency. Therefore, dysregulation of NO production has been considered as the cornerstone of endothelial dysfunction and much attention has been paid to the identification of pathways associated with suppression of NO synthase activity, the enzyme responsible for NO synthesis.

Dimethylarginines are by-products of the posttranslational methylation of arginine residues in proteins and they interfere with NO formation by inhibiting NO synthase which, in turn, leads to endothelial dysfunction. Asymmetrical dimethylarginine (ADMA) is the most potent inhibitor of NO synthase and has emerged as a novel biomarker of CV outcome and mortality among patients with intermittent and high CV risk, as well as in the general population [6, 7]. Since the details of the mechanisms accompanying this effect are unravelling, interest has started to divert towards symmetrical dimethylarginine (SDMA), the inactive congener of ADMA which has not been studied to a similar extent. SDMA is mainly excreted by kidneys and may provide a mechanistic link between renal dysfunction and CV disease. Elevated SDMA levels are associated with adverse outcomes in stroke [8], worse prognosis in patients referred for coronary angiography [9], and all-cause and CV mortality in the general population [10].

These findings suggest that SDMA may have an independent role in the pathogenesis of endothelial dysfunction and CV disease, by inhibiting both renal and cellular uptake of L-arginine, thus also potentially diminishing NO bioavailability [11, 12]. In addition, SDMA induces monocyte-mediated reactive oxygen species production [13] and has been described as a proinflammatory agent in chronic kidney disease [14]. In RA, the magnitude of the inflammatory response over time (cumulative inflammatory burden) rather than a single determination of inflammatory markers may be more important for the development and progression of vascular injury [15]. Despite emerging data in several conditions associated with atherosclerosis and CV disease, the role of SDMA in RA remains largely unexplored. The aim of the present study was to investigate whether cumulative systemic inflammatory burden and/or classical CV risk factors in RA associate with SDMA in a large well-characterized cohort of patients with long-standing RA.

2. Materials and Methods

2.1. Study Participants. We studied 201 patients with RA. The patients were part of the Dudley Rheumatoid Arthritis Comorbidity Cohort (DRACCO), a prospective study examining CV burden in RA. These individuals have participated in other studies of cardiovascular risk factors, and patient characteristics and detailed methods have been previously described [16, 17]. In summary, 201 out of 400 patients initially recruited in 2006 performed a follow-up visit in 2012. Seventy-eight patients had died since 2006 and the remaining 121 declined to participate for personal reasons. Exclusion criteria for the present study were confirmed acute coronary syndrome, evidence of chronic kidney disease, or serious psychiatric disorders according to their medical records. All patients met the 1987 revised RA criteria of the American College of Rheumatology [18]. The study received ethics approval from The Black Country Research Ethics Committee. All participants gave their written informed consent according to the Declaration of Helsinki.

2.2. Protocol for Baseline. Patients were evaluated after a 12-hour overnight fast and underwent a standardised clinical interview, physical examination, and detailed review of their medical history and hospital records. Height and weight were measured and the body mass index (BMI) was calculated (using a TANITA Body Composition Analyser BC-418). Disease activity score (DAS28) [19] and physical function using the Health Assessment Questionnaire (HAQ) [20] were recorded. Chart review with RA treatment and current therapy for other indications was performed. A blood sample was also obtained on the same day for the assessments of routine hematologic and biochemistry, lipid profile, fasting glucose, fasting insulin, and acute phase response. Insulin resistance was determined by using the Homeostasis Model Assessment Insulin Resistance (HOMA-IR) and Quantitative Insulin Sensitivity Check Index (QUICKI), as previously described [21, 22]. All biochemical tests were carried out in the Biochemistry Laboratory at Russells Hall Hospital, The Dudley Group NHS Foundation Trust, UK.

2.3. Protocol for Follow-Up Visit. Patients reported to the same clinical research facility as in the baseline assessment in 2012 after a 12-hour overnight fast. A venous blood sample was collected to assess SDMA levels. The SDMA assay is based on the method of competitive enzyme-linked immunoassays. The sample preparation includes the addition of a derivatization-reagent for SDMA coupling. During the incubation period, the target SDMA in the sample competes with the SDMA derivative (tracer) immobilised on the wall of the microtiter plates for the binding of the polyclonal antibodies. The SDMA in the sample displaces the antibodies out of the binding to the tracer. Therefore, the concentration of the tracer-bound antibody is inversely proportional to the SDMA concentration in the sample. The absorbance is measured at 450 nm and patient samples are read from a standard curve.

The intra-assay standard deviation was 7.5% and interassay was 6%. The lowest amount detected was 0.05 μmol/L.

2.4. Cumulative Inflammatory Burden. Detailed information on erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) from the baseline visit to the follow-up visit for each patient was used to calculate cumulative inflammatory burden. A quarterly measurement of CRP and ESR for each year the patient was in the study was used to calculate the area under the curve (AUC) for each parameter. ESR was measured using the StaRRsed Auto Compact blood sedimentation instrument (Mechatronics, Zwaag, Netherlands) and CRP was measured using the VITROS 5,1 FS Chemistry System (Ortho-Clinical Diagnostics, Rochester, NY, USA).

2.5. Statistical Analysis. Several of the variables being considered, including SDMA, followed skewed distributions. Where

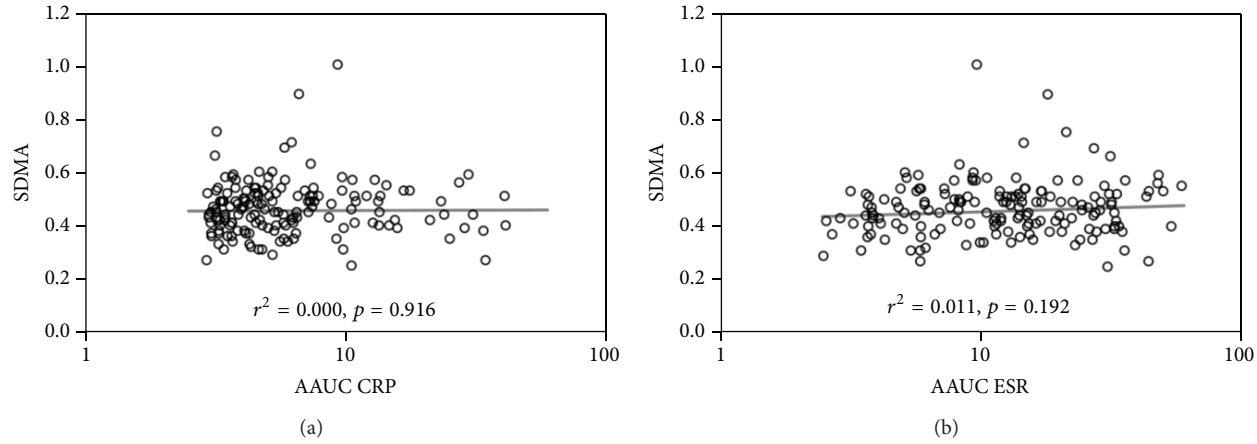

Figure 1: Graphic demonstration of the associations between SDMA and AAUCs of CRP (a) and ESR (b). Cumulative inflammation is plotted on a logarithmic scale, and the fit line and related statistics are based on the regression models in Table 2. SDMA: symmetric dimethylarginine, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, and AAUC: average area under the curve.

this was the case, $\log_2$-transformations were applied to the data prior to the analysis, to allow the use of parametric tests. AUCs for ESR and CRP data were calculated for the quarterly measurements made between 2006 and 2012. Since some patients had missing data at the start or end of the period, the AUCs were dependent on the length of time the data were available. For this reason, the resulting AUCs were divided by the total period that data were available for each patient to produce average AUCs (AAUCs).

The $\log_2$-transformed SDMA values were then compared across categorical factors using independent samples t-tests for dichotomous factors and one-way ANOVA where there were multiple categories. For the continuous factors, regression models were produced to quantify relationships with SDMA. All of the factors from the univariable analysis were then considered for inclusion in a multivariable regression model, using a forward stepwise entry method, in order to account for the potential effects of confounding.

All analyses were performed using IBM SPSS Statistics 22 (IBM Corp. Armonk, NY). Missing data was excluded on a per analysis basis, and $p < 0.05$ was deemed to be indicative of statistical significance.

3. Results and Discussion

3.1. Results. The characteristics of the participants at baseline and follow-up as well as the general demographics are summarised in Table 1. The majority of the patients were female with relatively low disease activity score and acute phase response, parameters indicating optimal RA control despite long disease duration.

Data were available for 201 patients, of whom 197 had SDMA measurements. After being $\log_2$-transformed, SDMA was found to closely follow a normal distribution, with the exception of 11 (6%) patients with SDMA values >1. These outliers were excluded from the data, in order to make parametric analysis valid and to prevent them from becoming excessively influential in the tests performed.

Univariable analysis of SDMA found only two factors to be significant predictors, namely, estimated glomerular filtration rate (eGFR) ($p = 0.024$) and creatinine ($p = 0.016$) (Table 2). For eGFR, the relationship was negative, with a one unit increase associated with a 0.3% (95% CI: 0.0%, 0.5%) reduction in SDMA. Creatinine, on the other hand, had a positive relationship with SDMA, with a twofold increase in the former associated with a 12.0% (95% CI: 2.2%, 22.8%) increase in the latter. Neither of the cumulative inflammatory markers was found to be significantly correlated with SDMA, with $p = 0.916$ for the AAUC of CRP and $p = 0.192$ for the AAUC of ESR (Table 2). This is demonstrated graphically in Figure 1.

Univariable analysis of categorical factors found no significant associations between SDMA, traditional CV disease risk factors, and treatment with steroids, methotrexate, tumour necrosis factor-alpha inhibitors, or antihypertensive agents (Table 3).

To consider the potential for confounding factors, multivariable analyses were performed, using forward stepwise regression models, with all of the variables in Tables 2 and 3 as potential covariates. Creatinine was the only factor entered into the final model, which was expected, since none of the other factors were significant in univariable analysis with the exception of eGFR, which was highly correlated with creatinine (Pearson's r: −0.83, $p < 0.001$).

3.2. Discussion. In this study, we assessed for the first time the relationship between SDMA and cumulative inflammatory burden and traditional CV risk factors in RA patients. Although SDMA has been reported as a surrogate marker of endothelial dysfunction in a number of conditions characterised by excess CV morbidity, no significant associations with disease-related inflammation, metabolic factors, and CV risk scores were detected in our RA population.

RA is characterized by a heightened inflammatory state and the chronic intense interaction occurring between

Table 1: Patient characteristics at baseline and at follow-up (Sandoo A, Dimitroulas T, Hodson J, Smith JP, Douglas KM, Kitas GD. Cumulative inflammation associates with asymmetric dimethylarginine in rheumatoid arthritis: a 6 year follow-up study. Rheumatology. 2014 Sep 3. [Ahead of print]. Reproduced with permission from Oxford University Press).

	Baseline (2006)	Follow-up (2012)
General characteristics		
Age (years)	61 (53–67)	67 (59–73)
Sex female *N* (%)	155 (77%)	155 (77%)
Body mass index (kg/M^2)	27 (24–30)	28 (24–32)
Disease characteristics		
Disease duration (years)	10 (4–18)	16 (11–25)
Rheumatoid factor positive *N* (%)	148 (74%)	148 (74%)
Anti-CCP positive *N* (%)	123 (61%)	123 (61%)
DAS28	4.0 (3.1–4.8)	3.1 (2.5–4.0)
HAQ	1.3 ± 0.9	1.6 ± 0.9
C-reactive protein (mg/L)	7.5 (4.3–16)	3 (2.9–8.5)
Erythrocyte sedimentation rate (mm/hr)	17 (8–30)	12 (5–23)
SDMA (μmol/L)	—	0.47 (0.40–0.53)
Cardiovascular disease risk factors		
Hypertension *N* (%)	132 (66%)	130 (65%)
Dyslipidemia *N* (%)	115 (57%)	158 (79%)
Insulin resistance *N* (%)	65 (32%)	53 (26%)
Diabetes *N* (%)	7 (4%)	21 (10%)
Global CVD risk scores		
Framingham Risk Score (%)	4 (1–8)	8 (5–13)
Reynolds Risk Score (%)	6 (3–13)	8 (3–14)
RA Medications		
Methotrexate *N* (%)	128 (64%)	122 (61%)
Prednisolone *N* (%)	58 (29%)	51 (25%)
NSAID's *N* (%)	47 (23%)	26 (13%)
Cyclooxygenase II inhibitors *N* (%)	14 (7%)	5 (3%)
Anti-TNFα therapy *N* (%)	20 (10%)	57 (28%)
Tocilizumab *N* (%)	—	3 (2%)
Cardiovascular medications		
Antihypertensive *N* (%)	81 (40%)	79 (39%)
Antihypercholesterolemic *N* (%)	33 (16%)	74 (37%)
Beta-blocker *N* (%)	32 (16%)	22 (11%)
Calcium channel blocker *N* (%)	26 (13%)	27 (13%)

Results are expressed as median (25th to 75th percentile values), mean ± standard deviation or number (percentage). DAS28 = disease activity score in 28 joints. Anti-TNFα = antitumour necrosis factor alpha; CCP = citrullinated protein antibody; HAQ = Health Assessment Questionnaire; NSAID's = nonsteroidal anti-inflammatory drugs; SDMA: symmetric dimethylarginine.

vasculature and inflammation could promote vascular changes conducive to increased CV risk. Thus, it is not surprising that morphological markers of atherosclerosis such as intima-media thickness have been correlated with the magnitude of systemic inflammation assessed by higher CRP values [23] and patients with elevated ESR are at increased risk of CV death compared to those with normal levels [24]. However, recent insights argue against a direct impact of the inflammatory process on vascular wall, demonstrating that systemic markers of inflammation do not correlate with changes in noninvasive vascular assessments of microvascular and macrovascular endothelial function characterizing different stages of atherosclerosis [4].

On the other hand, reports on the association between soluble biomarkers of endothelial dysfunction and indices of systemic inflammation have provided inconsistent results [25, 26]. Particularly for the dimethylarginines, conflicting associations between ADMA and the acute phase response have been reported, with some studies establishing positive correlations with CRP and ESR [17, 27] and others yielding negative results [28, 29]. These discrepancies can be attributed to different methodological approaches, cross-sectional study designs, inclusion of small and heterogeneous patient populations, and variations in disease duration, activity, and treatment. Importantly, the method of characterising the effect of inflammation on the vasculature may also influence

Table 2: Univariable analysis of continuous factors.

	SDMA	
	Coefficient (95% CI)	p value
BMI	−0.5% (−1.1%, 0.0%)	0.063
HR	−0.1% (−0.3%, 0.1%)	0.380
SBP	0.0% (−0.1%, 0.2%)	0.653
DBP	0.1% (−0.2%, 0.3%)	0.633
Log_2 Reynolds Risk Score	1.5% (−0.8%, 3.9%)	0.191
Log_2 Framingham Risk Score	−0.1% (−3.2%, 3.2%)	0.955
eGFR	−0.3% (−0.5%, 0.0%)	0.024*
Log_2 creatinine	12.0% (2.2%, 22.8%)	0.016*
Log_2 AAUC CRP	0.2% (−3.4%, 3.9%)	0.916
Log_2 AAUC ESR	2.0% (−1.0%, 5.0%)	0.192

Results from univariable regression models, with $\log_2$-transformed dependent variables. Coefficients were then antilogged, to represent the percentage change in the untransformed outcome for a unit increase in the factor. For $\log_2$-transformed factors, the coefficient represents the increase in the outcome for a twofold increase in the untransformed factor. *Significant at $p < 0.05$. BMI: body mass index (kg/M^2), HR: heart rate, SBP: systolic blood pressure, DBP: diastolic blood pressure, eGFR: estimated glomerular filtration rate, CRP: C-reactive protein, and ESR: estimated sedimentation rate.

Table 3: Univariable analysis of categorical factors.

	N	SDMA	
		Average	p value
High cholesterol			0.275
No	122	0.45 (0.44–0.47)	
Yes	64	0.47 (0.44–0.50)	
Hypertension			0.866
No	69	0.46 (0.44–0.48)	
Yes	117	0.46 (0.44–0.47)	
Insulin resistance			0.149
No	136	0.46 (0.45–0.48)	
Yes	50	0.44 (0.42–0.47)	
Smoking			0.957
Never	84	0.46 (0.44–0.48)	
Previously	78	0.45 (0.43–0.48)	
Currently	22	0.46 (0.42–0.50)	
Family history of cardiac events			0.506
No	102	0.46 (0.44–0.48)	
Yes	84	0.45 (0.43–0.47)	
MTX			0.837
No	69	0.46 (0.43–0.48)	
Yes	117	0.46 (0.44–0.48)	
Steroids			0.239
No	140	0.45 (0.44–0.47)	
Yes	46	0.47 (0.44–0.51)	
Anti-TNF			0.599
No	133	0.46 (0.44–0.47)	
Yes	53	0.46 (0.44–0.49)	
Antihypertensive			0.463
No	116	0.45 (0.44–0.47)	
Yes	70	0.47 (0.44–0.49)	

Data reported as "geometric mean (95% confidence interval)", with p values from independent samples t-tests/one-way ANOVA on $\log_2$-transformed values. Significant at $p < 0.05$.

the findings of studies. For example, the magnitude and chronicity of inflammation assessed with historical measures of cumulative inflammatory burden probably represents a more reliable marker of the total inflammatory burden to which an RA individual has been exposed during the course of the disease than a single reading of CRP or ESR [15]. To lend more support to this, cumulative inflammation shows better associations with noninvasive assessments of peripheral vascular function and morphology when compared with current inflammatory levels [5]; however, only a small number of studies have employed such an approach. Positive correlations between cumulative inflammatory burden and arterial stiffness have been reported in RA [30] and we recently found similar associations for ADMA [17].

Despite previous observations, we did not demonstrate any correlation between SDMA and cumulative inflammatory burden. One reason may be the effective control of inflammatory disease in our cohort. Other potential explanations include differences in biology between ADMA and SDMA. It also appears that dimethylarginines may promote endothelial dysfunction through different mechanisms even beyond NO synthase inhibition [31]. ADMA mediates oxidative stress in vasculature through endothelial NO synthase uncoupling and vice versa. Inflammation influences its metabolic pathways, resulting in elevated levels of ADMA, predominantly due to dimethylarginine dimethylaminohydrolase inhibition [32]. Inflammation and oxidative pathways represent important mechanisms accounting for the pathogenetic role of ADMA in CV disease. However, there has been limited understanding regarding the interplay between SDMA and inflammation. It is worth noting that SDMA remained unchanged in patients with acute bacterial infection during the course of the disease whilst changes in ADMA levels were noticed during the recovery phase of the infection [33].

SDMA has been associated with the expression of proinflammatory cytokines in patients with chronic kidney disease [14], but the aetiology of chronic inflammatory status characterising the uremic conditions differs from that of high-grade systemic inflammatory diseases such as RA. SDMA may exhibit proinflammatory properties associated with abnormal renal function contributing to the moderate inflammation present in the majority of uremic patients and corroborating the previously demonstrated increase in monocyte-mediated reactive oxygen species production [13]. However, the lack of association with inflammation in other populations may indicate its main biologic role as a uremic toxin and marker of renal function. In that respect, our results confirm the presence of a relationship between serum creatinine and SDMA in keeping with the findings from a meta-analysis by Kielstein et al. [34].

Traditional CV risk factors are more prevalent and poorly controlled in RA compared to general population, but they only partially account for the development of atherosclerosis

and increased CV risk [35]. It has been hypothesized that such risk factors operate differently in RA and general population, as some studies have reported that systemic inflammation provides positive modulation to the effects of the established factors in the vasculature and CV disease [36]. To lend more support, the appreciation that risk algorithms used for CV risk stratification in the general population underestimate the risk of future events in RA patients underlines the complexity of interrelations between traditional and disease-related risk factors in this condition [37]. Metabolic alterations such as insulin resistance, obesity, and aberrant lipid metabolism are more frequent in patients with RA and are associated with increased disease activity [38]. The association between cardiometabolic syndrome and RA may be reciprocal, with chronic low-grade inflammation characterising insulin resistance and high-grade RA-related systemic inflammatory activity reinforcing each other, resulting in a vicious circle promoting oxidative stress and vascular damage. In our cohort, CV risk factors were not found to influence SDMA levels and, to the best of our knowledge, this is the first study to investigate such associations, not only in RA, but also in other high-risk patient populations. Similar results have also been reported for ADMA in patients with RA [17], coronary artery [39], and cerebrovascular disease [40]. Whether SDMA represents a surrogate marker of endothelial dysfunction in RA remains to be determined. However, the lack of association with cumulative inflammatory burden and classic CV factors may suggest that, apart from a reliable indicator of renal function, its utility as mediator of vascular injury in RA may be limited.

Although the last decades have witnessed significant advances in treatment of RA, the mortality gap between RA individuals and general population has been widening, mainly due to CV disease [41]. Subsequently, CV risk prevention should constitute a key goal in management of this condition [42]. Besides clinical recommendations [43], there is an urgent need in identifying biomarkers to allow the early recognition of RA patients at increased risk for CV events and to facilitate individualised treatment strategies. Dimethylarginines have been shown to predict CV mortality and morbidity in the general [7, 44] population, but it is questionable whether these observations are applicable to RA where the pathogenesis of vascular damage may arise through different mechanisms triggered by inflammation and immune activation. It is, therefore, unlikely that a single biomarker could encompass the various processes involved in endothelial dysfunction in RA. However, disruption of NO metabolism is crucial, with ADMA and SDMA mediating, probably through different pathways, the deleterious effects of inflammation on the vascular wall and promoting endothelial injury and atherosclerosis. Such a process may be more pronounced in chronic high-grade inflammatory conditions such as RA, suggesting an important role for these molecules, whose potential to predict future CV events in RA patients remains to be investigated in large prospective studies with hard CV disease end points.

Our study has several strengths, as we investigated the association between SDMA and cumulative inflammation in a large real-life RA population with sufficient assessment and measurement of classical CV risk factors at baseline. Furthermore, repeated measurement of inflammatory markers made it possible to take variability in disease activity into account. In contrast, low disease activity in our patients may have contributed to the negative results. The absence of serial measurements of SDMA is another limitation, but we are not aware of any data suggesting fluctuation of SDMA levels over time. In addition, we excluded outliers with extremely high SDMA from the analysis to ensure the validity of our models. Finally, we did not find any associations between SDMA and antirheumatic treatment. However, large longitudinal studies with serial measurements of ADMA and/or SDMA are warranted to address the ability of dimethylarginines as indicators of potential endothelial function improvement following treatment with synthetic and biologic disease modifying drugs. Until today, only a small study showed reduction in ADMA levels achieved with biologic drugs [45] whilst others failed to demonstrate any changes in concentrations of ADMA and SDMA in RA and ankylosing spondylitis [46, 47].

4. Conclusion

In summary, our findings suggest that SDMA is independent of cumulative inflammatory load assessed by quarterly measurement of CRP and ESR for each year, as well as established CV risk factors in RA, but associations between SDMA and proinflammatory cytokines cannot be categorically excluded. Altered NO homeostasis is considered an important mechanism for vascular changes in RA and previous observations have revealed that reduced NO availability is associated with inflammation. However, the specific role of SDMA in the development of atherosclerosis and abnormal endothelial function in this population warrants further investigations.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Influence of Malondialdehyde and Matrix Metalloproteinase-9 on Progression of Carotid Atherosclerosis in Chronic Renal Disease with Cardiometabolic Syndrome

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Objective was to assess whether the concentration of malondialdehyde (MDA) as a marker of lipid peroxidation and serum concentration of matrix metalloproteinase-9 (MMP-9) are involved in the process of atherosclerosis in chronic kidney disease (CKD) patients nondialysis-dependent and those on peritoneal dialysis (PD), both with signs of cardiometabolic syndrome (CMS). Thirty CKD and 22 PD patients were included in a study. All observed patients were divided into three subgroups depending on the degree of atherosclerotic changes in the carotid arteries (CA). Severity of atherosclerotic changes in the CA was evaluated by ultrasonography. We confirmed significantly lower level of serum MDA throughout all the stages of atherosclerosis in PD patients compared with observed CKD patients ($P < 0.05$) and increased serum concentration of MDA and MMP-9 with the progression of severity atherosclerotic changes in both groups of patients. The multiple regression analysis revealed that MDA and MMP-9 are significant predictors of changes in IMT-CA CKD patients ($P < 0.05$) and plaque score on CA in these patients ($P < 0.05$). The results suggest that MDA and MMP-9 could be mediators of CKD-related vascular remodeling in CMS.

1. Introduction

Cardiovascular disease is one of the major causes of morbidity and mortality worldwide, especially among patients of chronic kidney disease (CKD) [1, 2]. There is an increasing evidence of cardiometabolic syndrome (CMS) involvement in CKD [3, 4], but a causal relationship has not been proven. Cardiometabolic syndrome is a cluster of metabolic abnormalities combining obesity with 2-3 risk factors which include insulin resistance, hypertension, and high triglyceride or low high density lipoprotein (HDL) serum levels. It also increases the risk of cardiovascular disease (CVD) and type 2 diabetes [5].

It is believed that atherosclerotic changes in blood vessels in chronic renal diseases contribute significantly to the high cardiovascular morbidity and mortality. Morphological and functional abnormalities of the endothelium are considered as prodromal stage of atherosclerosis and early marker of CVD [6] that facilitate the progress of atherosclerosis [7, 8] and contribute to the development of hypertension through the enhancement of vascular resistance. On the other hand, arterial calcifications are a significant risk factor for cardiovascular mortality in the general population. There is a strong evidence supporting the view that atherosclerosis is a disease characterized by low-level vascular inflammation [9–11]. Inflammation, inflammatory action of local stimuli such as products of the oxidation process and glycation end products, oxidative stress, and degradation of extracellular matrix (ECM) change vasculature in terms of development of atherosclerosis.

Renal disease is associated with a graded increase in oxidative stress (OS) markers even in early CKD [12]. Oxidative stress can accelerate renal injury progression and contribute increasing cardiovascular risk. Some studies

have documented that peritoneal dialysis is associated with decreased levels of oxidative stress and inflammatory markers compared to haemodialysis [13]. A small number of trials have been carried in order to determine associations between oxidative stress with vascular structure and function with equivocal results [14, 15]. On the other hand, uncontrolled expression of matrix metalloproteinases (MMPs), enzymes that degrade extracellular matrix (ECM), can result in tissue damage and the development of a number of destructive diseases such as arthritis, atherosclerotic plaque rupture, aortic aneurysm, and progression of tumors [16, 17].

However, the relevance of malondialdehyde as a marker of oxidative stress which is generated by peroxidation of unsaturated fats as well as the role of matrix metalloproteinase-9 in atherosclerosis progression in patients (pts) with chronic kidney disease (CKD) not yet on dialysis compared to patients on peritoneal dialysis is less known, particularly with respect to cardiometabolic syndrome.

The aim of this paper was to examine whether the serum concentration of malondialdehyde and matrix metalloproteinase-9 is involved in the process of atherosclerosis in patients with CKD not yet dialysis-dependent and those on peritoneal dialysis (PD) with signs of CMS.

2. Patients, Materials, and Methods

2.1. Study Population. This cross-section study was conducted at the Clinic for Nephrology, Clinical Center University in Sarajevo, from June 2014 through December 2014. Fifty-two adult patients with CMS and chronic kidney disease were included in the study. The subjects were divided into CKD patients not yet dialysis-dependent (30 pts; eGFR < 60 > 15 mL/min/1.73 m^2) and patients on peritoneal dialysis for >6 months (22 pts). Antioxidants had not been taken by any subjects in the two groups. All PD patients underwent 4 to 5 dialysis changes with 2 liters of dialysis solution. The control group consisted of 20 age- and sex-matched healthy subjects.

Subjects who had an episode of peritonitis within the previous 3 months and patients with evidence of malignancy, autoimmune disease or chronic liver disease, active infection, history of cardiovascular or peripheral vascular disease, and diabetes and recent treatment with iron were excluded from the study.

Depending on the degree of severity of atherosclerotic changes in the carotid arteries all observed group patients were divided into three subgroups (without atherosclerosis (AS0), moderate atherosclerosis (AS1/2), and severe atherosclerosis (AS3)).

Kidney function was assessed by using estimated glomerular filtration rate (eGFR). Estimated glomerular filtration rate was performed using the MDRD (abbreviated Modification of Diet in Renal Disease equation GFR) [18]. Body mass index (BMI) was calculated as the ratio of body weight in kilograms and the square of height in meters (BMI = kg/m^2). Body weight for BMI in PD patients is measured with dry abdomen (without dialysate solution). The blood pressure (BP) was measured with mercury sphygmomanometer after 15 minutes of rest, according to recommendation by the British Hypertension Society [19]. Hypertension was defined as systolic BP ≥ 140 mmHg and diastolic BP ≥ 90 mmHg or use of antihypertensive medications.

Informed consent was obtained from all participants and the local ethics committee approved the study.

2.2. Measurement of Serum Concentration of Malondialdehyde (MDA) and Matrix Metalloproteinase-9 (MMP-9). All serum samples were stored at −80°C until they were measured.

The concentration of malondialdehyde (MDA) was analyzed at the Center for Cytogenetics and Molecular Medicine at the Medical Faculty in Sarajevo using a competitive enzyme immunoassay test (ELISA), which was performed with commercial kit for the assessment of the overall level of MDA (manufacturer: USCN Life Science Inc., US-CEA597GE). Reading of the results is carried out at 450 nm on a plate reader STAT FAX 2100, USA. The measurement concentration of MAD was expressed in nanograms per milliliter (ng/mL).

The concentration of the enzyme matrix metalloproteinase-9 (MMP-9) in serum was quantified by ELISA at the Clinic for Immunology University Clinical Centre Sarajevo, according to manufacturer's instructions (R&D Systems, Inc., RD- DMP900). Reading of the results is carried out by spectrophotometry at 450 nm (reader BIOTEK ELX50), with the correction wavelength at 540 nm or 570 nm. The measurement concentration of MMP-9 was expressed in nanograms per milliliter (ng/mL).

2.3. Ultrasound Examination of the Carotid Arteries. The severity of carotid artery atherosclerosis was evaluated using the mean common carotid artery (CA), intima media thickness (IMT), and plaque score (PS). Carotid ultrasonography was used to evaluate the mean IMT and the PS. High-resolution B mode and color Doppler and pulse Doppler ultrasonography of both carotid arteries were performed with an ultrasound scanner (Wall-Track system: W-T, Maastricht, the Netherlands) equipped with a 7.5-MHZ linear array transducer. Measurement was done by the same angiologist who was not familiar with the clinical status of the study patients. Patients were examined in the supine position with the head tilted backwards. After the carotid arteries were located by transverse scans, the probe was rotated 90° to obtain and record a longitudinal image of the anterior and posterior walls. The high-resolution images of the walls of the bilateral CA, internal carotid arteries (ICA), and carotid bulbs were examined according to recommendations of the American Society of Echocardiography Carotid Intima Media Thickness Task Force [20]. The IMT was defined as the distance between the leading edge of the lumen-intima echo and the leading edge of the media-adventitia echo in plaque-free area. At least three measurements (A, B, and C) were taken over one centimeter length of each wall segment CA, and these measurements on both sides were collected and divided to obtain the mean IMT.

Table 1: Basal characteristics of monitored patients.

	Control subjects (20)	CKD and PD pts (52)	P	CKD pts with eGFR < 60 > 15 mL/min/1.73 m^2 (30)	PD pts (22)	P
Age (years)	57.8 ± 14.2	59.8 ± 161	>0.05	63.6 ± 15.1	54.7 ± 16.2	>0.05
Smokers (yes)	35%	46%	>0.05	52%	38%	>0.05
SBP (mmHg)	124 ± 8.68	140.5 ± 19.65	<0.01	135.3 ± 20.44	147.6 ± 16.4	<0.05
DBP (mmHg)	79.8 ± 5.5	90.5 ± 11.22	<0.05	83.7 ± 10.67	90.7 ± 11.21	<0.05
Hemoglobin (g/L)	146.3 ± 11.82	117.3 ± 22.48	<0.01	126.4 ± 23.92	104.7 ± 12.2	<0.01
Albumins (g/L)	37.6 ± 1.76	34.4 ± 6.02	>0.05	36.8 ± 5.83	31.1 ± 4.59	<0.01
CRP (mg/L)	2.8 ± 1.96	9.2 ± 8.81	<0.01	9.8 ± 9.15	8.4 ± 8.48	>0.05
Cholesterol (mmol/L)	5.2 ± 1.37	5.7 ± 0.81	>0.05	4.9 ± 1.35	5.8 ± 1.26	>0.05
Triglycerides (mmol/L)	1.9 ± 0.15	2.0 ± 1.19	>0.05	1.8 ± 0.79	2.4 ± 1.52	>0.05
Uric acid (μmol/L)	272.9 ± 53.1	362.7 ± 104.2	<0.01	364.7 ± 120.8	360 ± 79.9	<0.01
BMI (kg/m^2)	22.3 ± 2.2	26.6 ± 2.95	>0.05	26.9 ± 3.7	25.7 ± 2.2	>0.05
MDA (ng/mL)	24.9 (12.3–26.9)	37 (24.1–47.7)	<0.01	37 (27–47.1)	35.9 (23.7–47.7)	>0.05
MMP-9 (ng/mL)	321.9 (24–370.9)	419.4 (350.9–447)	<0.01	420 (390.1–442)	390 (350.9–464)	>0.05

Results are expressed as mean ± standard deviation or median and interquartile range (25%–75%); pts: patients; CKD: chronic kidney disease; PD: peritoneal dialysis; SBP: systolic blood pressure; DBP: diastolic blood pressure; CRP: C-reactive protein; BMI: body mass index.

The PS was calculated by adding the maximal thickness in millimeters of plaques in each segment on both sides (A + B + C + thickness of the contralateral carotid artery plaques). The length of individual plaques was not considered in determining the PS.

The presence of atherosclerosis in CCA is estimated as recommended by the Mannheim consensus about atherosclerosis [21]:

(1) without atherosclerosis (AS0): IMT less than 80% of the reference interval (RI), age- and gender-adjusted, with RI values obtained from previously published studies of monitoring, using the same ultrasound procedures;

(2) mild atherosclerosis (AS1): IMT > 80% of the RI;

(3) moderate atherosclerosis (AS2): the presence of carotid plaque, with no significant stenosis (PSV < 125 cm/s);

(4) severe atherosclerosis (AS3): the presence of carotid plaque with threatening stenosis (PSV > 125 cm/s).

2.4. Statistical Analysis. All data were expressed as the mean ± SD or as median and interquartile range. The distribution of variables was tested by the Kolmogorov-Smirnov and/or Shapiro-Wilk test. Student's t-test was used to compare the means of variable with normal distribution. Kruskall-Wallis test was used for statistical evaluation of more than 3 groups. The difference in median with interquartile range between two groups was analyzed by the Mann-Whitney test. Pearson's test was used to correlate data with normal distribution and Spearman's test for data with a skewed distribution. A multiple regression analysis was applied to define the independent connection serum concentration of MDA and MMP-9 with ultrasound parameters of CA.

The significant independent variables were determined according to their standardized effect, defined as regression coefficient/standard error of the regression (β). P values of < 0.05 were considered statistically significant. All statistical calculations were performed with the SPSS 16 software (version 16.0, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Demographic Data. The average age of respondents was 59.84 ± 5.16 years and was not different from the age of the control group. There was also no difference in age and smoking in CMS patients with CKD without the need for dialysis treatment and those on peritoneal dialysis.

All monitored CKD and PD patients were overweight, while BMI of control group patients was within the limits that are considered healthy (between 20.1 and 24.5). In our study, all observed CKD patients, dependent or not dependent on dialysis treatment, were hypertensive (BP ≥ 140/90 mm Hg), but significantly higher values of both systolic and diastolic blood pressure were registered in the group of patients on PD treatment compared to other CKD patients. Although there were no significant intergroup differences in serum triglyceride levels, all groups of patients had an elevated level of serum triglycerides (above the recommended value of 1.7 mmol/L).

Basal characteristics of the anthropometric profile, blood pressure values, metabolic pattern, and serum concentration of MDA and MMP-9 in the whole group of CKD and PD subjects with CMS and in two subgroups of CKD with and without dialysis (PD) are presented in Table 1.

Table 2: The serum concentration of MDA and MMP-9 in different patient subgroups according to atherosclerotic stage.

	AS0	AS1/2	AS3	P
		CKD		
MDA (ng/mL)	24.1 (18.8–27)	40.3 (31.9–45.6)	74.3 (68.2–76.3)	<0.01
MMP-9 (ng/mL)	320 (250–350)	425.2 (405.5–439.5)	462.4 (458.1–464.2)	<0.01
		PD		
MDA (ng/mL)	20.6 (10.4–31.3)	35.6 (28.3–39.1)	53.9 (49.8–63.2)	<0.01
MMP-9 (ng/mL)	369.4 (350.5–432.6)	374.8 (346.4–397.7)	469.3 (464–480.6)	<0.01

3.2. Analysis of MDA and MMP-9 Serum Concentration in the Dialysis and Nondialysis Patient Subgroups According to the Stage of Atherosclerosis. Depending on the stages of atherosclerosis our data showed significant changes in serum concentration of MDA and MMP-9 between the different subgroups of CKD nondialysis-dependent patients, as well as in patients on peritoneal dialysis (Table 2).

3.3. Analysis of MDA and MMP-9 Serum Concentration between CKD and PD Patients According to the Stage of Atherosclerosis. The level of serum MDA through all the stages of atherosclerosis was significantly lower in PD patients compared to observed CKD nondialysis-dependent patients (in AS1/2 35.6 (28.3–39.1) versus 40.3 (31.9–45.6) ng/mL; $P < 0.05$ and in AS3 53.9 (49.8–63.2) versus 74.3 (68.2–76.3) ng/mL; $P < 0.05$). There were no significant differences in serum concentration of MDA in the monitored PD and CKD patients without expressed atherosclerotic changes (AS0 20.6 (10.4–31.3) versus 24.1 (18.8–27) ng/mL; $P > 0.05$) (Figure 1).

The level of serum concentration of MMP-9 was significantly different in PD patients compared to CKD nondialysis-dependent patients. Significantly lower level of this biomarker is registered in the stage AS1/2 of atherosclerosis in PD patients (374.8 (346.4–397.7) ng/mL) compared to the CKD patients treated conservatively (425.2 (405.5–439.5) ng/mL) ($P < 0.05$), whereas in stage AS3 serum MMP-9 concentration was significantly higher in PD patients in comparison with other CKD patients ($P < 0.05$). Such a relationship but lower concentration of MMP-9 in the serum was present in stage AS0 in both groups (Figure 2).

Significant correlation was confirmed between serum concentration of MDA and IMT-CA in all observed patients ($r = 0.859$; $P < 0.01$) (Figure 3), as well as between serum concentration of MDA and value of plaque score (rho = 0.869; $P < 0.01$) (Figure 4).

Significant relation was also confirmed between serum concentration of MMP-9 and IMT-CA ($r = 0.762$; $P < 0.01$) (Figure 5) and between serum concentration of MMP-9 and value of plaque score in all observed CKD and PD patients ($r = 0.785$; $P < 0.01$) (Figure 6).

In multiple regression model serum concentrations of MDA and MMP-9 were significant predictors of IMT-CA in all monitored CKD and PD patients ($P < 0.05$), as well as plaque score on carotid arteries in these populations ($P < 0.05$). This model is able to explain 70% of the variance in the results of IMT ($R^2 = 0.765$) and about 79% ($R^2 = 0.786$) of the variation that occurs with the plaque score on carotid arteries in chronic kidney disease (Table 3).

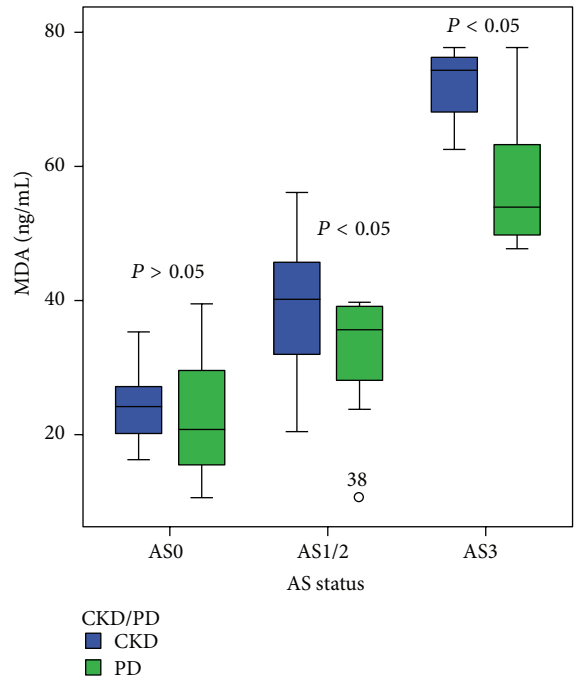

Figure 1: The relationship between serum concentrations of MDA in CKD nondialysis-dependent patients and PD patients according to the stage of atherosclerotic changes in the carotid arteries. Data are presented as median with interquartile range. Bars show the maximum and minimum value, while the square and its central bar show median and interquartile range. Note: AS: atherosclerosis.

4. Discussion

Chronic kidney disease is regarded as a prooxidant and low-grade inflammation state. The degree of intracellular and extracellular oxidative stress is related to the severity of renal failure [22]. It was also noted that tissue damage caused by lipid peroxidation plays an important role in the developmentof various diseases including atherosclerosis, which is associated with high cardiovascular morbidity and

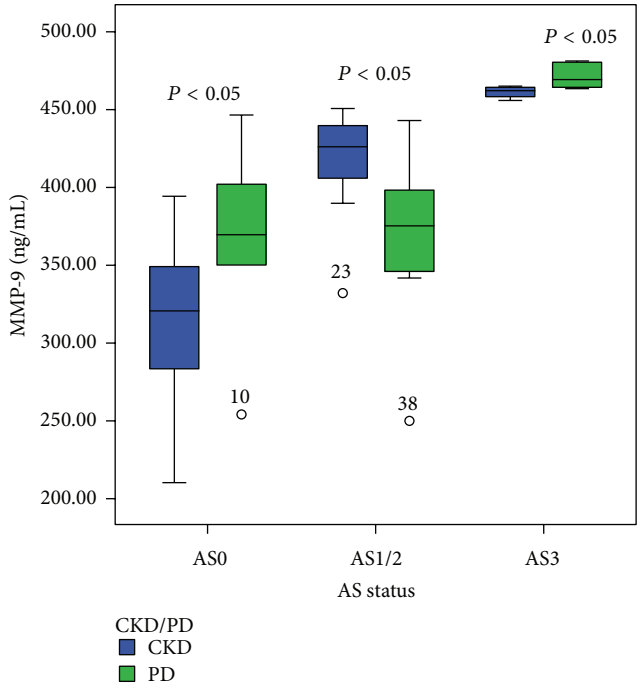

Figure 2: The relationship between serum concentrations of MMP-9 in CKD nondialysis-dependent patients and PD patients according to the stage of atherosclerotic changes in the carotid arteries. Data are presented as median with interquartile range. Bars show the maximum and minimum value, while the square and its central bar show median and interquartile range. Note: AS: atherosclerosis.

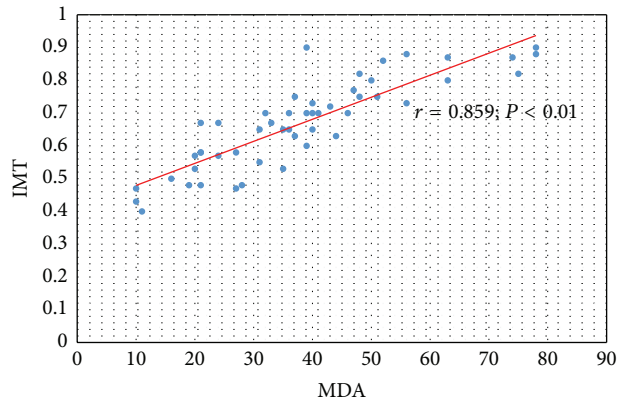

Figure 3: The relation between serum concentration of MDA and IMT-CA in CKD and PD patients.

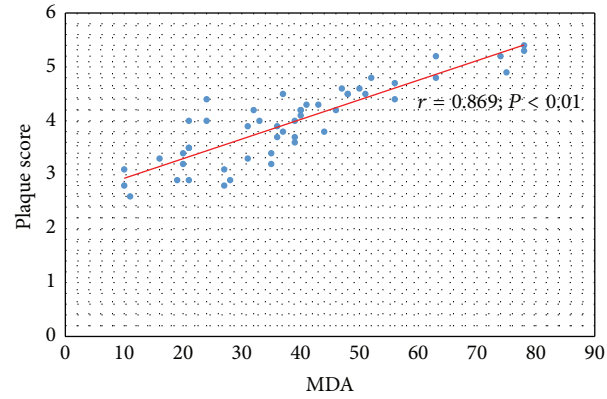

Figure 4: The relation between serum concentration of MDA and value of plaque score in CKD and PD patients.

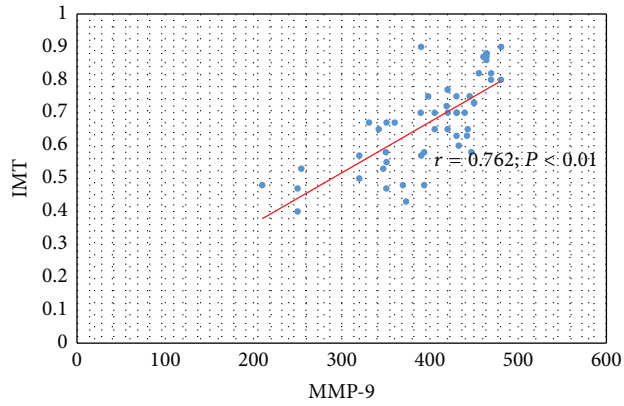

Figure 5: The relation between serum concentration of MMP-9 and IMT-CA in CKD and PD patients.

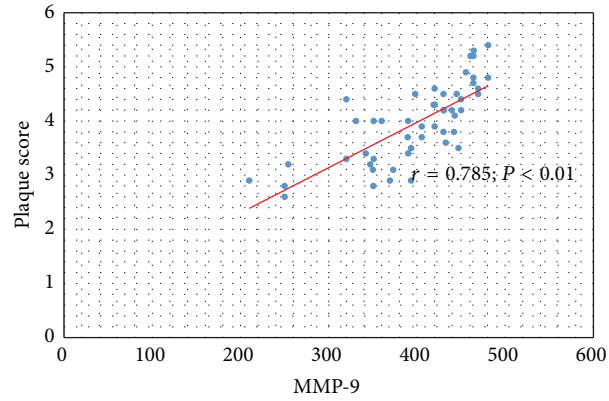

Figure 6: The relation between serum concentration of MMP-9 and value of plaque score in CKD and PD patients.

mortality in CKD [23, 24]. In recent years a few studies were published dealing with the status of oxidative stress in relation to various disorders that accompany chronic renal disease and renal replacement therapy [25–29].

In the present paper we demonstrated that the serum concentration of MDA did not statistically differ in CKD patients with CMS treated conservatively and patients undergoing peritoneal dialysis. However, it was observed that serum concentration of MDA progressively increases with severity of atherosclerotic changes in the carotid arteries within both groups of patients. On the other hand, we have also shown significant changes in serum concentration of MDA between the different subgroups of CKD and PD patients according to the status of atherosclerotic changes in the carotid arteries. The highest levels of serum MDA were in stage AS3 in both groups of patients. Significant

Table 3: Multiple regression model of study biomarkers and their relation with indicators of carotid atherosclerosis in observed CKD and PD patients.

	B	SE	Beta	*t*	*P* value	CI 95% lower–higher
MDA (ng/mL)	0.005	0.001	0.675	6.595	**0.000**	0.004–0.007
MMP-9 (ng/mL)	0.001	0.000	0.254	2.480	**0.017**	0.000–0.001
Dependent variable: IMT						
MDA (ng/mL)	0.029	0.004	0.683	6.941	**0.000**	0.020–0.037
MMP-9 (ng/mL)	0.003	0.001	0.255	2.592	**0.013**	0.001–0.005
Dependent variable: plaque score						

SE: standard error; CI: confidence interval: IMT: intima media thickness; MDA: malondialdehyde; MMP-9: matrix metalloproteinase-9.

correlation was confirmed between serum concentration of MDA with IMT-CA and plaque score in all study patients. In addition, our results indicate that the level of serum MDA through all the stages of atherosclerosis was significantly lower in PD patients in comparison with observed CKD patients treated conservatively, which can be attributed to the influence of preserved residual renal function (RRF) and the absence of trusted signs of inflammation. Since peritoneal dialysis is intrinsic type of dialysis through the peritoneum as biocompatible membrane, this result also suggests the possible influence of peritoneal dialysis on oxidative stress.

A few studies reported increased OS among PD patients, but other prooxidants (TBARS, TAC) were measured, associated with hypoalbuminemic state or loss of residual renal function (RRF), and without comparing it with nondialysis CKD patients [25, 27, 30]. Khaira et al. [31] found that PD patients have markedly impaired endothelial function as documented by impaired flow mediated dilatation of brachial artery with higher serum concentration of OS markers. Furthermore, Raju et al. [29] confirmed significant increase in serum MDA in hemodialysis patients, compared with the same patients before they had started the hemodialysis treatment, which could be attributed to a bioincompatibility of dialysis membrane and diffusion of hydrophilic compounds to the dialysate and influx of endotoxin from the dialysate. These factors lead to activation of macrophages and production of reactive oxygen species (ROS) and also a loss of antioxidants during hemodialysis sessions [26]. All the above factors cause an increase in production of free radicals, peroxidation of lipids, and further rise in serum MDA level after episodes of dialysis [32]. Our results suggest that oxidative stress is one of the factors that mediate the relationship between CKD, atherosclerosis, and CMS.

Activity of MMP-9 is highly associated with the progression of CKD, diabetes, and coronary arterial disease [33, 34]. This MMP is secreted by inflammatory cells in the adventitia or smooth muscle cells in the media. The degraded elastic fiber induces calcium deposition, which in conjunction with altered vascular structure is associated with vessel stiffening [35, 36]. In CKD arterial stiffening increases cardiac afterload, left ventricular hypertrophy, reduces coronary artery perfusion and myocardial ischaemia, and increases pulse pressure that promotes atheroma formation and vascular remodeling [37].

In our study, no significant difference was found in the serum concentration of MMP-9 between CKD patients treated conservatively and PD patients, but the significant increase was found in the value of this biomarker within both groups with progression of atherosclerotic process. These findings are in line with the findings of Addabbo and associates, who have demonstrated that MMP-9 levels strongly correlated with carotid atherosclerosis burden irrespective of other factors in early, moderate, and advanced CKD [38].

Our data also showed the decreased serum concentrations of MMP-9 in the stage AS1/2 of atherosclerosis in PD patients compared with CKD patients without dialysis treatment. Possible reason of such decrease is unknown. However, it is our belief that the decrease in MMP-9 concentration in AS1/2 stage of PD patients might occur due to the impact of peritoneal dialysis on MMP-9 serum concentration. MMP-9 was significantly higher in stage AS3 in PD patients compared with CKD patients treated conservatively. To our knowledge, the impact of peritoneal dialysis on the concentration MMP-9 has not yet been clarified. Some authors have demonstrated that the hemodialysis process leads to a reduction in plasma concentration of MMP-9 in some patients [38].

A significant correlation of MMP-9 serum concentration with CA-IMT and plaque score was found in all monitored patients in this study. Also, in multiple regression model we confirmed a significant independent association of MDA and MMP-9 with these ultrasonography parameters characterizing arterial wall and atherosclerotic changes of carotid arteries.

The need for identification of risk factors and serum markers of atherosclerosis in the process of early detection and prediction of risk for cardiovascular disease has attracted a lot of attention in recent years. The significantly lower levels of MDA in PD-CMS patients in comparison with CKD-CMS patients not yet dialysis-dependent and its increase with atherosclerosis progression, as well as obtained higher values of MMP-9 during progression of atherosclerosis especially in PD patients, could be a new contributing factor of our study.

However, this study has certain limitations, such as the small number of respondents and a cross-sectional design of study. A question on the periodontal disease status of those patients was not included, although periodontal disease and kidney disease are highly associated and periodontal disease is alone associated with increases in MMP-9 [39].

Whether the longitudinal profile of MDA and MMP-9 in CKD nondialysis-dependent patients and PD patients, both with signs of CMS, provides additional information on the predictive power of these biomarkers for the progression of atherosclerosis remains a question for testing in future longitudinal studies with a larger number of patients.

5. Conclusion

The results suggest that MDA and MMP-9 could be mediators of CKD-related vascular remodeling in CMS. The study data also suggest that that factors mediating relationship between cardiometabolic syndrome, chronic kidney disease, and atherosclerosis can include malondialdehyde and MMP-9.

Abbreviations

MDA: Malondialdehyde
MMP-9: Matrix metalloproteinase-9
eGFR: Estimated glomerular filtration rate
CKD: Chronic kidney disease
PD: Peritoneal dialysis
CVD: Cardiovascular disease
CMS: Cardiometabolic syndrome
TBARS: Thiobarbituric acid reactive substances
TAC: Total antioxidant capacity
ROS: Reactive oxygen species
AGE: Advanced glycosylated end products
OS: Oxidative stress
ECM: Extracellular matrix
IMT: Intima media thickness
PS: Plaque score
pt: Patient
CA: Carotid artery
AS: Atherosclerosis
PSV: Peak systolic velocity.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Senija Rašić and Damir Rebić contributed equally to the paper.

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Research Article

Urinary Malondialdehyde Is Associated with Visceral Abdominal Obesity in Middle-Aged Men

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The purpose of the present study was to investigate multiple anthropometric parameters used to evaluate obesity, particularly visceral abdominal fat area, and various metabolic parameters including malondialdehyde (MDA) as an oxidative stress marker. We evaluated various measures of obesity, including body mass index (BMI), waist circumference (WC), sagittal abdominal diameter, fat percentages using dual-energy X-ray absorptiometry, visceral fat area (VFA), subcutaneous fat area, multiple biomarkers related to metabolic disease, and urinary MDA, in 73 asymptomatic middle-aged men who were not severely obese. We examined relationships between multiple measures of obesity, metabolic markers, and urinary MDA levels and evaluated associations between VFA and urinary MDA. In the visceral obesity group, γ-glutamyl transferase (GGT), uric acid, and urinary MDA levels were significantly higher than in the nonvisceral obesity group ($P = 0.008$, $P = 0.002$, and $P = 0.018$). Urinary MDA ($r = 0.357$, $P = 0.002$) and uric acid ($r = 0.263$, $P = 0.027$) levels were only significantly positively correlated with VFA among measures of obesity. Urinary MDA, serum GGT, and serum CRP were significantly positively associated with VFA ($P = 0.001$, $P = 0.046$, and $P = 0.023$, resp.), even after adjusting for BMI and WC.

1. Introduction

Obesity is associated with hypertension, dyslipidemia, metabolic syndrome, and type 2 diabetes [1, 2]. In particular, visceral abdominal fat accumulation is closely linked to insulin resistance and cardiovascular disease (CVD) [3, 4]. Obesity is usually defined by body mass index (BMI), waist circumference (WC), or body fat percentage; however, these measures are limited in their ability to distinguish visceral obesity, which plays a key role in the pathogenesis of cardiometabolic syndrome. Although their average BMI is low, Asians demonstrate higher fat percentages compared with Caucasians of equivalent BMI and have higher prevalence of diabetes and CVD [5, 6]. Alanine aminotransferase (ALT) [7], γ-glutamyl transferase (GGT) [8], uric acid [9], and C-reactive protein (CRP) [10] are suggested to be metabolic markers associated with hepatic dysfunction, oxidative stress, and inflammation.

Oxidative stress refers to an imbalance between oxidants and antioxidants on a cellular or individual level [11]. Many studies found higher oxidative stress levels in obese than nonobese people [12–14], which may be due to several potential mechanisms including chronic inflammation [15–17], hyperglycemia [18], or impairment of antioxidant defense

systems [19, 20]. Malondialdehyde (MDA) has been recognized as an important indicator of lipid peroxidation that is generated as an end product from oxidative degradation of polyunsaturated fatty acids [21, 22]. Urinary MDA is especially useful as a noninvasive test for monitoring oxidative stress.

Studies that evaluate the correlation between various measures of obesity and metabolic parameters, focusing on visceral obesity, are scarce. Therefore, the present study was designed to investigate the associations between multiple anthropometric parameters of obesity, particularly those relating to visceral abdominal fat area, and various metabolic parameters in middle-aged men who were not severely obese. We were particularly interested in the association between visceral obesity and metabolic parameters, especially urinary MDA, after adjusting for BMI and WC.

2. Material and Methods

2.1. Study Subjects and Ethics. The study was approved by the Institutional Review Board at Pusan National University Yangsan Hospital, and informed written consent was obtained from all subjects before participating. Study subjects included 80 asymptomatic men between the ages of 40 and 60 years who visited the health promotion center in Pusan National University Yangsan Hospital. Subjects with a history of malignancy or a cardiovascular event and subjects receiving medication for acute diseases, such as myocardial infarction and angina pectoris, were excluded. Subjects with low body weight (BMI $< 18.5\,kg/m^2$) or severe obesity (BMI $\geq 30.0\,kg/m^2$) were also excluded. Seventy-three middle-aged men were enrolled in this study.

2.2. Measurements. Following an overnight fast, blood samples were obtained from an antecubital vein between 08:00 and 09:00 a.m. ALT, GGT, and uric acid concentrations were measured using the Hitachi 7600 Analyzer (Hitachi Co., Ltd., Tokyo, Japan) by an enzymatic colorimetric method. Low-density lipoprotein and high-density lipoprotein were measured with Toshiba TBA200FR using a direct measurement method and triglycerides (TG) were measured using lipase, GK, GPO, and POD with a glycerol blank. CRP was measured using a Behring BN II nephelometer (Dade Behring, Marburg, Germany).

Height and weight were measured down to the first decimal place, and BMI was calculated as body weight (kg) divided by the squared height (m). Using a tape measure, WC was measured from the half-way point between the lower line of the last rib and the upper line of the iliac crest when a subject exhaled and was measured down to the first decimal place. Each subject's sagittal abdominal diameter (SAD) was measured in a supine position with legs extended using a portable sliding-beam caliper (Holtain Ltd., Dyfed, Wales, UK). The vertical abdominal length was measured in a supine position by letting the subject slightly lift the midsection up and inserting the fixed lower arm of the portable sliding-beam caliper at the location of iliac crest of pelvis (space between L4 and L5), letting the subject inhale deeply and slowly exhale, and lowering the upper arm of the portable sliding-beam caliper down until it touched the abdomen without pressing it. Measurements were taken to the nearest tenth of a cm [23].

Abdominal fat was assessed from computed tomography (CT) scans taken at the L4-L5 level. Abdominal fat was defined as the area corresponding to the pixel range from −190 to −30 Hounsfield units [24]. The visceral and subcutaneous abdominal adipose tissue areas were measured. The fat inside the peritoneum was considered visceral adipose tissue, and the fat between the dermis and muscle fascia was considered subcutaneous adipose tissue. Whole body fat composition was obtained using dual-energy X-ray absorptiometry (DXA) (Hologic Inc., Bedford, MA, USA).

Data on alcohol intake and smoking habits were obtained by interview. Subjects were divided into two groups by the amount of alcohol consumption: nondrinker 0–180 g/week and drinker >180 g/week. Smoking status was classified as nonsmoker or (former or current) smoker. Both diet and physical activity were assessed due to their possible effects on insulin sensitivity. Diet was monitored by using a semi-quantitative food frequency questionnaire [25] and physical activity was assessed using the International Physical Activity Questionnaire [26]. Physical activity levels are expressed in MET-minute.

2.3. Urine MDA. Urinary MDA was measured with high performance liquid chromatography (HPLC). For the quantification of urinary MDA by HPLC [27], 3.0 mL (1%) phosphoric (V) acid, 0.4 mL ultrapure water, and 0.6 mL of sample or standard (1–125 μM MDA) were added to a screw-capped test tube and mixed thoroughly. Then, 1.0 mL of 0.67% thiobarbituric acid was added to all tubes and kept in a 95°C water bath for 1 hour. After incubation, the tubes were placed in an ice bath. Then, the tubes were centrifuged for 15 minutes. The supernatant was filtered before being applied to a Zorbax Eclipse XDB-C8 (I.d. 4.6 × 250 mm, 5 μm, Agilent). Measurements were made with a UV-Vis detector (Agilent 1100 series HPLC system, USA) at 532 nm. The mobile phase was 0.05 M potassium phosphate buffer (pH 6.8) with methanol (50 : 50, v/v). The flow rate was 0.5 mL/min. MDA and creatinine were analyzed in two voided specimens. Urinary MDA levels were expressed as μmol/g creatinine, averaged, and used for analysis.

2.4. Statistical Analysis. Descriptive data were expressed as the mean value (±SD) or number (%). The 73 subjects were divided into two groups: the visceral obesity (VO) group and nonvisceral obesity (non-VO) group. The visceral obesity group consisted of the subjects whose CT visceral fat area (CT-VFA) was $\geq 100\,cm^2$. The Shapiro-Wilk test was used to test the normality of the variables. To compare the means of two groups, we used the two-sample t-test or the Mann-Whitney U test for continuous variables depending on the normality of the variables. For the categorical variables, the Chi-square test was used to verify the group effect. Correlations between variables were tested using Spearman's correlation coefficients. Finally, multiple regression analysis

Table 1: General characteristics of subjects.

Characteristics	Value
Number of participants	73
Age (y; mean ± SD)	51.2 ± 5.8
Height (cm; mean ± SD)	171.4 ± 5.3
Weight (kg; mean ± SD)	71.8 ± 7.7
BMI (kg/m^2; mean ± SD)	24.4 ± 2.1
Waist circumference (cm; mean ± SD)	86.3 ± 6.7
Abdominal VFA (cm^2; mean ± SD)	100.8 ± 35.2
Abdominal SFA (cm^2; mean ± SD)	127.6 ± 50.2

BMI: body mass index; VFA: visceral fat area; SFA: subcutaneous fat area.

was performed to investigate the relationship among the variables considering GGT, UA, CRP, TG, and urinary MDA levels as dependent variables and abdominal VFA as the independent variable after adjusting for BMI and WC. The analysis was conducted using SPSS version 18.0 for Windows (SPSS Inc., Chicago, IL). Statistical significance was accepted for P values <0.05.

3. Results

3.1. Clinical Characteristics of Subjects. The general characteristics of the study subjects are presented in Table 1. The overall average age was 51.2 years and mean BMI was 24.4 kg/m^2. Mean abdominal VFA was 100.8 cm^2.

3.2. Differences between the Characteristics of Patients in the Two Study Groups. No differences in age, smoking status, alcohol habits, physical activity, or dietary intake were observed between the VO group and non-VO group (Table 2).

The VO group had significantly higher BMI, WC, and SAD than the non-VO group ($P = 0.009$, $P = 0.002$, and $P = 0.003$, resp.), and DXA-measured trunk fat and total fat percentages were also significantly higher in the VO group ($P = 0.005$ and $P = 0.014$, resp.). However, there were no differences in DXA-measured upper arm fat and lower leg fat percentages. CT-measured subcutaneous fat area was similar between the two groups ($P = 0.117$). GGT and uric acid levels were higher in the VO group ($P = 0.008$ and $P = 0.002$, resp.), and TG levels were marginally higher in the VO group ($P = 0.063$). Urinary MDA levels were significantly higher in the VO group ($P = 0.018$).

3.3. Correlations between Obesity Measures, Metabolic Parameters, and Urinary MDA. BMI, WC, and DXA-measured total fat percentages were significantly positively correlated with ALT, GGT, CRP, and TG (Table 3), but not significantly correlated with uric acid or urinary MDA. However, urinary MDA ($r = 0.357$, $P = 0.002$) and uric acid ($r = 0.263$, $P = 0.027$) levels were only significantly positively correlated with VFA among the measures of obesity.

3.4. Multiple Regression Analysis of Abdominal VFA and Metabolic Parameter and Urinary MDA. In all subjects, the association between VFA and GGT ($\beta = 0.23$, $P = 0.028$) and CRP ($\beta = 0.278$, $P = 0.01$), as well as urinary MDA ($\beta = 0.362$, $P = 0.001$), remained significant after adjusting for BMI (Table 4). These associations also remained after adjusting for BMI and WC (GGT: $\beta = 0.207$, $P = 0.046$; CRP: $\beta = 0.245$, $P = 0.023$; urinary MDA: $\beta = 0.349$, $P < 0.001$). On the other hand, the associations between VFA and uric acid or TG were not significant after adjusting for BMI and WC.

4. Discussion

In the present study, we investigated associations between abdominal VFA and common clinical metabolic biomarkers in middle-aged men without morbid obesity. In addition, we were particularly interested in the correlation between urinary MDA, a known oxidative stress marker, and abdominal visceral adiposity. We demonstrated that urinary MDA, GGT, and CRP were significantly positively associated with VFA, even after adjusting for BMI and WC.

Oxidative stress is considered a crucial factor because this is an early instigator of metabolic syndrome [12] and a contributor to the development of major obesity-related comorbidities such as CVD [13]. MDA is a biomarker derived from lipid peroxides and that is considered useful marker of oxidative marker [28]. Previously published data showed that participants with a high VFA (≥100 cm^2) were more likely to have high plasma MDA levels, which is consistent with our findings, although our study examined urine MDA level [29]. We checked urinary MDA as oxidative stress marker because that is noninvasive test. In our data, urine MDA is a marker of oxidative stress in obese people, especially those with visceral obesity.

We wondered if previously known cardiometabolic biomarkers as well as urinary MDA levels have been connected with various measures of obesity and specifically those laboratory markers that reflect visceral obesity. We confirmed that urinary MDA levels were related to abdominal visceral fat area after adjusting for BMI and WC, which are typically used to evaluate clinical obesity. Urinary MDA levels can indicate inflamed adipose tissue. Further study is required to determine the clinical utility of urinary MDA levels.

Serum uric acid was higher in the VO group than the non-VO group and showed a positive correlation with VFA; however, after adjusting for BMI and WC, there was no association with VFA. On the other hand, GGT and CRP demonstrated a significant association with VFA even after adjusting for BMI and WC. Previous studies showed GGT was strongly associated with metabolic syndrome, which is a combined expression of metabolic disorders including abdominal obesity [8]. In several studies, CRP levels already showed positive and significant correlations with body fat mass and VFA measured by CT [30, 31] in men. These results suggested that CRP levels can reflect inflammation by visceral adiposity. Previously published data demonstrated a highly significant association between smoke exposure and MDA [32, 33]; however, in the present study, the levels of urinary MDA in smokers were not elevated compared with nonsmokers.

Table 2: Characteristics of patients in the two study groups.

	Nonvisceral obesity (n = 38)	Visceral obesity (n = 35)	P value
Sociodemographic parameters			
Age (years)	50.3 ± 6.1	52.2 ± 5.5	0.185
Smoking status			0.162
Nonsmoker	24 (60.0)	16 (40.0)	
Smoker	14 (42.4)	19 (57.6)	
Alcohol consumer	30 (50.8)	33 (49.2)	0.770
Activity (METS/week)*	1602.3 ± 2000.8	907.1 ± 932.2	0.268
Dietary parameters			
Energy intake (Kcal/kg/day)	28.9 ± 5.0	28.0 ± 5.1	0.475
Protein intake (g/kg/day)	1.2 ± 0.3	1.2 ± 0.3	0.800
Fat intake (g/kg/day)	0.7 ± 0.2	0.7 ± 0.2	0.543
Carbohydrate (g/kg/day)	4.6 ± 0.7	4.3 ± 0.8	0.163
Anthropometric parameters			
Body mass index (kg/m^2)	23.8 ± 1.8	25.1 ± 2.2	**0.009**
Waist circumference (cm)	83.9 ± 5.1	88.8 ± 7.4	**0.002**
Sagittal abdominal diameter (cm)	18.3 ± 1.4	19.8 ± 2.6	**0.003**
DXA-measured fat			
Upper arm fat (%)	24.8 ± 4.6	26.4 ± 4.5	0.139
Lower leg fat (%)	23.6 ± 4.0	25.1 ± 4.6	0.148
Trunk fat (%)	27.6 ± 3.9	30.6 ± 5.1	**0.005**
Total fat (%)	25.6 ± 3.4	27.8 ± 4.2	**0.014**
CT-measured abdominal fat area			
VFA (cm^2)*	74.9 ± 16.4	128.8 ± 27.9	<**0.001**
SFA (cm^2)*	120.2 ± 42.1	135.7 ± 57.3	0.117
Metabolic parameters			
ALT (IU/L)*	31.2 ± 17.0	35.1 ± 16.0	0.156
GGT (IU/L)*	46.2 ± 28.7	99.5 ± 99.5	**0.008**
Uric acid (IU/L)*	6.2 ± 1.0	7.1 ± 1.2	**0.002**
CRP (mg/dL)*	0.10 ± 0.07	0.18 ± 0.25	0.147
LDL-cholesterol (mg/dL)	138.1 ± 35.6	137.9 ± 32.9	0.981
HDL-cholesterol (mg/dL)*	53.2 ± 10.7	49.3 ± 10.9	0.154
TG (mg/dL)*	132.4 ± 72.0	173.2 ± 97.0	0.063
Oxidative stress parameter			
Urinary MDA* (μmol/g creatinine)	1.56 ± 0.85	2.08 ± 1.16	**0.018**

Data are expressed as the mean ± SE or the number (%).
P value by 2-sample t-test or chi-square test.
VFA: visceral fat area; SFA: subcutaneous fat area; DXA: dual-energy X-ray absorptiometry; ALT: alanine aminotransferase; GGT: γ-glutamyl transferase; CRP: C-reactive protein; LDL: low-density lipoprotein; HDL: high-density lipoprotein; TG: triglyceride; MDA: malondialdehyde.
One MET is roughly equivalent to 1 kcal/min for a person weighing 60 kg.
* P value by Mann-Whitney U test.

This study has several limitations, which include a relatively small sample size and a study population limited to relatively healthy middle-aged men. However, the use of this population limits the effects of various unmeasured confounding factors. Unfortunately, we did not measure blood MDA concentration and could not evaluate their correlation with urinary MDA and VFA. In addition, the study was conducted using a cross-sectional design, and thus further studies are required. Nonetheless, we believe our findings are meaningful because this study represents a new attempt to evaluate multiple anthropometric parameters evaluating obesity including visceral abdominal fat area and various metabolic parameters. Furthermore, we suggest that urinary MDA levels may be useful as a marker of inflamed adipose tissue.

Table 3: Correlations between obesity indices, metabolic parameters, and urinary malondialdehyde levels.

	BMI	WC	Fat percent	VFA	SFA
ALT	**0.394***	**0.305***	**0.277***	0.154	**0.384***
GGT	**0.355**†	**0.335**†	**0.380**†	**0.389**†	**0.376**†
Uric acid	0.112	0.168	0.105	**0.263***	0.066
CRP	**0.343**†	**0.346**†	**0.290***	**0.326**†	**0.345**†
TG	**0.255***	**0.274***	**0.257***	**0.254***	**0.233***
Urinary MDA	0.081	0.031	−0.013	**0.357**†	0.005

ALT: alanine aminotransferase; GGT: γ-glutamyl transferase; CRP: C-reactive protein; TG: triglyceride; MDA: malondialdehyde; BMI: body mass index; VFA: visceral fat area; SFA: subcutaneous fat area; WC: waist circumference.
P value by Spearman's correlation.
$^{*}P < 0.05$, $^{\dagger}P < 0.01$.

Table 4: Multiple regression analysis of associations between abdominal visceral fat area and metabolic parameters.

Abdominal VFA	Adjusted BMI				*P* value	Adjusted BMI and WC				*P* value
	F	*B*	SE	β		*F*	*B*	SE	β	
GGT	14.03†	0.106	0.047	0.230	**0.028***	10.70†	0.095	0.047	0.207	**0.046***
Uric acid	12.43†	5.703	3.240	0.185	0.083	10.23†	4.680	3.196	0.152	0.148
CRP	15.34†	53.703	20.231	0.278	**0.010***	11.296†	47.434	20.396	0.245	**0.023***
TG	11.17†	0.035	0.043	0.086	0.419	9.008†	0.028	0.042	0.069	0.507
Urinary MDA	19.37†	12.321	3.301	0.362	<**0.001**†	14.389†	11.867	3.259	0.349	**0.001**†

GGT: γ-glutamyl transferase; CRP: C-reactive protein; TG: triglyceride; MDA: malondialdehyde; BMI: body mass index; VFA: visceral fat area; WC: waist circumference.
$^{*}P < 0.05$, $^{\dagger}P < 0.01$.

5. Conclusion

Urinary MDA, serum GGT, and serum CRP were significantly positively correlated with VFA, even after adjusting for BMI and WC in middle-aged healthy men. Further study is needed to confirm the validity of urinary MDA as a marker of inflamed adipose tissue.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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